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Inflammatory bowel disease: mechanisms, redox considerations, and therapeutic targets.

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/139303> since 2016-10-06T14:20:52Z

Published version:

DOI:10.1089/ars.2012.4530

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(Article begins on next page)

This is the author's final version of the contribution published as:

Biasi F; Leonarduzzi G; Oteiza PI; Poli G. Inflammatory bowel disease: mechanisms, redox considerations, and therapeutic targets..
ANTIOXIDANTS & REDOX SIGNALING. 19 (14) pp: 1711-1747.
DOI: 10.1089/ars.2012.4530

The publisher's version is available at:

<http://online.liebertpub.com/doi/abs/10.1089/ars.2012.4530>

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<http://hdl.handle.net/2318/139303>

Comprehensive Invited Review

INFLAMMATORY BOWEL DISEASE: MECHANISMS, REDOX CONSIDERATIONS AND THERAPEUTIC TARGETS

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Running head: oxidative stress and inflammation in the gut

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Word count: 17992 (excluding references, tables, figure legends and abbreviations)

Reference numbers: 300

Grayscale illustrations: 6

Color illustrations: 5 (online 5: Figs. 2, 3, 4, 6, 8)

Tables: 2

“Reviewing Editors: Ida Casorelli, Deanna Gibson, Liudmila Korkina, Patricia Lin, Hironori Nakagami, Donal P O’Leary, Balakrishnan S. Ramakrishna and Thomas R Ziegler”

ABSTRACT

Significance: Oxidative stress is thought to play a key role in the development of intestinal damage in Inflammatory Bowel Disease (IBD), because of its primary involvement in intestinal cells' aberrant immune and inflammatory responses to dietary antigens and to the commensal bacteria. **Recent advances:** During the active disease phase, activated leukocytes generate a wide spectrum of pro-inflammatory cytokines, but also excess oxidative reactions, which markedly alter the redox equilibrium within the gut mucosa, and maintain inflammation by inducing redox-sensitive signaling pathways and transcription factors. Moreover, several inflammatory molecules generate further oxidation products, leading to a self-sustaining and auto-amplifying vicious circle, which eventually impair the gut barrier. **Critical issues:** The current treatment of IBD consists of long-term conventional anti-inflammatory therapy and often leads to drug refractoriness or intolerance, limiting patients' quality of life. Immune modulators or anti-TNF α antibodies have recently been used, but all carry the risk of significant side effects and treatment poor response. **Future Directions:** Recent developments in molecular medicine point to the possibility of treating the oxidative stress associated with IBD, by designing a proper supplementation of specific lipids to induce local production of anti-inflammatory derivatives, as well as developing biological therapies that target selective molecules (i.e. NF- κ B, NADPH oxidase, prohibitins or inflammasomes) involved in redox signaling. The clinical significance of oxidative stress in IBD is now becoming clear, and may soon lead to important new therapeutic options to lessen intestinal damage in this disease.

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I. INTRODUCTION

IBD comprises a group of idiopathic chronic inflammatory intestinal conditions of which Crohn's disease (CD) and ulcerative colitis (UC) are the two main categories.

IBD is considered to be a chronic intermittent inflammatory process, in which active disease alternates with variable periods of remission, the evidence of tissue lesions being differentially localized in CD and UC.

Intestinal tissue in CD is characterized by patchy transmural inflammation, with the presence of lesions along the whole tract of the gut mucosa. Multiple granulomas, especially localized in the ileo-cecal or ileo-colic areas, and extra-intestinal complications are common features in these patients. UC patients show diffuse inflammation limited to the superficial layers of the colonic mucosa, and relapse at least once within ten years from diagnosis. In addition, they are prone to developing pancolitis with megacolon and colon carcinoma, as well as extra-intestinal complications. As far as the etiopathogenesis is concerned IBD appears to depend on the interaction between genetic alterations and environmental stressors that induce an aberrant response by innate, adaptive and tolerogenic immunity of the intestinal mucosa to dietary antigens and/or commensal bacteria.

Chronic inflammation in IBD is characterized by massive leukocyte infiltration of the gut. Upon activation, these cells produce a wide spectrum of pro-inflammatory cytokines but also an excessive amount of reactive oxygen (ROS) and nitrogen (RNS) species. Importantly, the marked and sustained alteration of redox equilibrium within the gut mucosa towards an excess of oxidative reactions, i.e. a condition of oxidative stress, plays a pivotal role in the expression and the progression of IBD. Oxidative stress maintains active inflammation within the intestinal mucosa by inducing redox-sensitive signaling pathways and transcription factors. Conversely, several inflammatory reactions and molecules generate further amounts of ROS, leading to a self-sustaining and auto-amplifying vicious circle that in turn leads to structural and functional impairment of the gut barrier, and affects its responsiveness to commensal flora and pathogens present in the lumen.

The highest incidence rates and prevalence of IBD and UC have been reported in the United States and Northern Europe. The incidence of IBD is now also increasing in other regions of Europe

and Asia, in direct correlation to economic development and industrialization. Other factors that influence the incidence rate of the disease are gender, age and ethnicity. CD is more frequent in females, while UC is much more frequent in males. The age peak for CD is 20-30, while it is 30-40 for UC. Different susceptibilities to IBD have been reported for the Jews, as well as for the whites and African Americans (high), Hispanic and Asian Americans (both increasing), but with marked variations induced by migration (49). Concerning the likely combination of genetic and environmental factors in IBD pathogenesis, variants of multiple genes involved in microbe recognition, lymphocyte activation, cytokine signaling and intestinal epithelial defense, could make a given population more susceptible to environmental attack (190).

This review, after a rapid survey of the current understanding of the mechanisms that regulate intestinal barrier integrity and function, as well as its pathologic alterations during the development of IBD, focuses on the pathogenetic roles played by the multiple redox changes that occur during the development of this disease process. The main indications and suggestions for targeted therapy of IBD that arise from the recent molecular studies and in particular from the redox reconsideration of the disease are also examined and discussed.

II. MOLECULAR MECHANISMS OF INTESTINAL BARRIER DYSFUNCTION IN IBD

In IBD, both the structure and the function of the intestinal barrier are compromised, with a loss of tolerance to normal dietary components and/or excessive response to pathogens, which all contribute to amplify the overall inflammatory process.

II A. Alteration of the Apical Junctional Complex

The cytokines interferon γ (IFN γ) and tumor necrosis factor α (TNF α), which are central mediators of intestinal inflammation, are able to damage epithelial barrier functions independently of their pro-apoptotic action. These cytokines have been shown to disassemble tight junctions (TJs) of apical junctional complex (AJC) and to enhance paracellular permeability, by promoting internalization and cellular redistribution of their junctional adhesion molecule (JAM)-A, occludin

and claudins1, but not of zonula occludens-1 (ZO-1) or adherens junction (AJ) proteins in T-84 cells (32).

A dramatic loss of JAM-A expression has been found both in CD and UC, and also in dextran sodium sulfate (DSS)-induced experimental colitis in mice. Consistently, JAM-A null mice, in which colitis was induced by DSS treatment, showed a marked increase in epithelial permeability and inflammatory cytokine production (272).

Redistribution of claudins has also been observed in IBD: while claudins-3 and -4 seem to be reduced, or almost entirely redistributed to the basolateral surface of the epithelium, the expression of claudin-2 increased predominantly in cells along inflamed crypts of both UC and CD patients. Interestingly, claudin-2 was up-regulated in T-84 cells by Interleukin (IL)-13, which is a key effector of the T helper (Th)2 response and is produced in large amounts in the lamina propria of UC patients (205). In these individuals, IL-13 has been found to induce apoptosis of colonic epithelial cells and to lower transepithelial resistance by increasing paracellular permeability (92).

It is still unclear whether damage to the transmembrane proteins that are components of junctions should be considered a cause or a consequence of intestinal inflammation. Under inflammatory conditions, as in IBD patients, endocytosis of junctional molecules increases, and intracellular redistribution may cause a breakdown of the protective barrier. This process may enhance production of inflammatory infiltrate and of cytokines, which further contribute to amplifying epithelial barrier damage.

II B. Impairment of intestinal antimicrobial peptides

The intestinal mucosa is covered by a thick layer of secreted mucus containing antimicrobial molecules, which protect the mucosal surface against microbial invasion. It has been suggested that a possible cause of IBD is a defect in mucus secretion that can expose the mucosa not only to pathogenic microbes, but also to assault by commensal bacteria that become pathogens, and contribute to chronic inflammatory response. Several mucin glycoproteins and antimicrobial peptides,

such as defensins, cathelicidins, lactotransferrin, and lysozymes, are highly effective in the host defense.

Mucins (MUCs), trefoil factors (TFFs) and resistin-like molecule β (RELM β), are produced by goblet cells in varying proportions throughout the human gastrointestinal tract. Up to 20 different mucin genes have been identified: MUC2 was the first human secretory mucin to be identified and widely studied. Mice with missense mutations in the *MUC2* gene or *MUC2*-deficient animals spontaneously develop increased susceptibility to colitis (90, 270).

A significant down-regulation of *MUC2* in CD and *MUC12* gene expression has been found in colon tissue in both CD and UC (171). *MUC1*, *MUC3* and *MUC19* have also been identified as possible susceptibility genes for IBD (165, 231).

TFF3 interacts with MUC2 by enhancing the stability of the mucin layer, thus protecting epithelial cells from intestinal stressors (124). *TFF3*-deficient mice show a higher susceptibility to DSS-induced colitis. Conversely, *TFF3* overexpression increases resistance to intestinal damage in mice (126). RELM β is also strongly expressed in intestinal goblet cells by colonization with normal enteric bacteria, and in mice with gastrointestinal infections or IBD. It is involved in the induction of CD4⁺ T cell-dependent adaptive immune response (179).

Paneth cells control bacterial attack of the host mucosa, regulating bacterial numbers by secreting various defensins, as well as lysozyme and secretory phospholipase A₂ (Plase A₂). Defensins provide an important antimicrobial function, because of their ability to regulate the adherence of specific bacteria to the intestinal epithelium. In humans, two α -defensins secreted under normal conditions by Paneth cells have been identified, namely human α -defensins (HD)-5 and HD-6 (130). Reduced expression of HD-5 and HD-6 by Paneth cells has been observed in ileal mucosa from CD patients, with no changes in lysozyme or secretory Plase A₂ expression (283).

The importance of human β -defensins (HBDs) in IBD pathogenesis is still unclear. HBD-1 is constitutively expressed in small amounts in normal colonic epithelial cells. HBD-1 has been found to be decreased in the inflamed mucosa of IBD patients. In contrast, HBD-2 was strongly induced in the inflamed mucosa of UC, but not CD, patients (210, 284). Defensin production is under the control

of the molecules involved in commensal and pathogenic bacteria recognition, namely nucleotide-binding oligomerization domain (NOD)-like receptors and Toll-like receptors (TLRs) (see below). Alterations to HD and HBD expression are reported to be associated with caspase activation and recruitment domain 15 (CARD15)/NOD2 mutations, which in turn are thought to be responsible for an increased susceptibility to CD (278, 284).

Cathelicidins are antimicrobial peptides, synthesised by neutrophils, macrophages and intestinal epithelial cells (IEC), which modulate microbial growth, wound healing, and inflammation; they counteract gastrointestinal pathogens, including several *Helicobacter* strains, *Shigella*, *Salmonella* and *Candida albicans* (102). It is unknown whether they are associated with IBD; however, recent experimental studies have revealed a new role for these peptides in UC therapy. Administration of a synthetic mouse cathelicidin significantly prevented the development of DSS-induced ulcerative colitis in mice (249).

The response to commensal and pathogens bacteria and viruses of this antimicrobial peptide is mainly mediated by selective activation of the TLR-nuclear factor- κ B (NF- κ B) pathway (124). In fact, *MUC2* has been shown to have NF- κ B binding sites in the promoter. A number of *Lactobacillus* strains up-regulate HBD-2 via induction of NF- κ B, activator protein 1 (AP-1) and mitogen-activated protein kinase (MAPK) signaling pathways (224). Recently, because of TFF3 ability to inhibit TNF α production, TLR4 and NF- κ B in a well-established model of trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice, TFF3 has been hypothesized to be a potent agent in controlling IBD (258).

II C. Impairment of receptor-mediated microbial recognition

The different dietary antigens are recognized by pathogen recognition receptors (PRRs), which discriminate among pathogenic bacteria, commensal bacteria, and nutrients. PRRs recognize conserved molecular motifs known as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). PRRs include membrane bound TLRs, the RNA helicase family of receptors, i.e. retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and cytoplasmic NOD-like receptors (NLRs). TLRs recognize bacteria-derived lipopolysaccharide (LPS), flagellin or

unmethylated cytosine nucleotide next to guanine separated by a phosphate (CpG); RIG-I and the melanoma differentiation-associated gene 5 (MDA5) belong to the RNA helicase family of receptors, and are primary sensors of RNA viruses; NLRs recognize bacteria-derived peptoglycans (285).

1) Toll-like receptors and activation of redox-sensitive transcription factors

TLRs are expressed in different cell types of the gastrointestinal tract, including IEC, macrophages, Paneth and goblet cells, enteroendocrine cells, and immune cells, such as dendritic cells and CD4⁺ T cells. They selectively recognize different pathogens: lipopeptides are recognized by TLR2, viral-derived double-stranded RNA by TLR3, LPS is detected by TLR4, TLR5 recognizes flagellin derived from *Salmonella* species, and TLR9 is activated by CpG DNA dinucleotides.

Most TLRs, except for TLR3, activate the major signaling pathway dependent on the common adaptor protein, known as myeloid differentiation primary response gene 88 (MyD88), which is recruited through its Toll-IL-1-resistance (TIR) domain, to interact with the TIR domain of TLRs. This process culminates in the activation of NF- κ B, a redox-sensitive transcription factor playing a major role in the inflammatory process. Alternatively, TLR3 and TLR4 may signal in a MyD88-independent pathway, recruiting another adaptor molecule, TIR domain-containing adaptor-inducing IFN β (TRIF); this adapter leads to activation of the transcription factor interferon regulatory factor 3 (IRF3) and results in activation of type I IFNs, particularly IFN β (290). Further, TLR2 and TLR4 appear to be implicated in protecting the epithelial barrier against pathogens, stimulating the expression of intestinal cell HBD-2 by activating the c-Jun N-terminal kinase (JNK)-AP-1 pathway (277) and by activating autophagy processes; TLR2 and TLR4 accumulate on the autophagosome where they recognize microbial cell-wall components (5, 183). Notably, AP-1 is another transcription factor whose activation depends on the cellular and tissue redox state.

Sustained hyper-activation of TLRs occurs in the tissue lesions of most inflammatory disorders, and thus dysregulated TLR-mediated cell signaling, often resulting in hyper-activation of NF- κ B and other redox-sensitive transcription peptides, may also contribute to the pathogenesis of IBD (89).

2) Retinoic acid inducible gene I (RIG-I)-like receptors

As far as RLRs are concerned, RIG-I and MDA5 are cytosolic receptors belonging to the RNA helicase family, which are primary sensors of RNA viruses in the early phase of infection, and are constitutively expressed in the IEC. Interestingly, it has been shown that RIG-I is closely associated with the actin cytoskeleton in cultured IEC, and localizes predominantly at the apical-lateral cell junctions of both polarized human colorectal adenocarcinoma CaCo-2 cells and healthy human colon and small intestine. Besides playing a role in immune recognition, RIG-I has been suggested to be involved in regulating cellular motility and migration (176).

RLRs induce IFN production in response to virus infection, through the common adaptor protein interferon- β promoter stimulator-1, which signals interferon regulatory factors IRF3, IRF7, and NF- κ B. RLRs interact with different signal transduction pathways, such as just another kinase (JAK) and the signal transducer and activator of transcription (STAT), amplifying the IFN response, and they provide signaling crosstalk to enhance TLR expression and function (159).

3) NOD-like receptors and their modulation by reactive oxygen species

NLRs are emerging as crucial regulators of the inflammatory response to commensal microflora in the gut. NLRs are cytoplasmic proteins characterized by the presence of N-terminal protein-protein interaction domains (such as CARD, pyrin and baculovirus inhibitor of apoptosis protein repeat domains), a central conserved NOD domain, and the carboxy-terminal leucine-rich repeats (LRRs), which are involved in microbial sensing. Among the different NLRs (23 members in humans), NOD1 and NOD2 have been most widely studied in the intestine. NOD1 is ubiquitously expressed in intestinal cells, including IEC, whereas high levels of NOD2 are constitutively expressed in phagocytes and dendritic cells, and are induced in Paneth cells of the small intestine by inflammatory stimuli. These NLRs recognize distinct PAMPs: NOD1 recognizes γ -glutamyl-meso-diaminopimelic acid, while NOD2 recognizes muramyl dipeptide. They are actively involved in the recognition and activation of inflammatory and immune responses against intestinal pathogenic

bacteria, such as *Enterobacteriaceae*, *Helicobacter pylori*, and *Listeria monocytogenes*. Upon activation, NOD1 and NOD2 form cytoplasm platforms called “NOD signalosomes”, which activate MAPKs and NF- κ B, by recruiting the receptor-interacting protein 2 (RIP2), and producing pro-inflammatory cytokines (e.g. IL-8 and IL-1 β) (74). NOD2-dependent NF- κ B activity appears to be closely related to the perturbation of actin dynamics in cells (145). Through NF- κ B activation, NOD2 induces the release of antibacterial molecules produced in Paneth cells, such as HD.

Another set of NLRs responds to different stimuli to form protein complexes known as inflammasomes, which are involved in the activation of pro-caspase-1. Caspase-1 is considered to be pro-inflammatory, because it activates immature forms of the IL-1 family (IL-1 β , IL-18 and IL-33) and induces IL-1 secretion. There are different groups of inflammasomes, the main ones being nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 1 and 3 (NLRP1, NLRP3), and IL-1 β converting enzyme-protease activating factor (IPAF).

Of interest, ROS are crucial in up-regulating NLRP3 inflammasome activity. Most of the danger associated molecular patterns (DAMPs) listed above can activate phagocytic cells, with a consequent overproduction of ROS. For example, enhanced generation of ROS has been observed after macrophage-like THP1 cell exposition to asbestos and silica; NLRP3 activation is triggered by ROS produced by up-regulated NADPH oxidase (61) (See Sections VII B and C).

Inflammasomes may play an important role in IBD, especially because of their ability to activate pro-inflammatory caspases. Recent reports have hypothesized a relationship between NLRPs dysregulation and IBD. Activation of NLRP1 has been observed in metaplastic Paneth cells in the colon of IBD patients (237). Furthermore, NLRP3 inflammasome has been found crucial in inducing experimental colitis in mice: *NLRP3*^{-/-} mice developed a markedly less severe clinical picture following DSS treatment (19). NLRP3 was found to be overexpressed both in the experimental model of TNBS-induced colitis in mice, and in the damaged mucosa of human CD patients. However, the precise role of NLRP3 inflammasome in IBD is still under debate. Recent reports have shown that NLRP3 inflammasome-induced cytokine production might, under specific conditions, confer protection against experimental colitis (95, 296).

In conclusion, consistently impaired signaling by TLRs and NLRs takes place during the development of IBD, with consequent enhancement and endorsement of inflammatory reactions within the gut wall (see the schematic representation in Figure 1).

II D. The role of the endothelium in IBD progression

IBD development comprises alternate phases of tissue damage and tissue repair. The latter process implies neoangiogenesis, then the synthesis of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), by macrophages and lymphocytes recruited in the inflammatory areas. Endothelial cells of the inflamed capillaries also contribute to the production of angiogenic factors.

Intestinal endothelial cells express CD40 and CD40L proteins, which belong to the TNF receptor superfamily, and which have co-stimulatory activity for immune cells, such as T cells, and for non-immune cells. CD40 pathway activation leads to the amplification of immune and inflammatory responses. In both ulcerative colitis and Crohn's disease, CD40 and CD40L are overexpressed, especially in severely inflamed mucosa (52).

Bacterial antigens that can penetrate the epithelial barrier may reach the endothelium, where they activate PRR signaling: various TLRs and both NOD1 and NOD2 have also been found expressed on the surface of endothelial cells, and are up-regulated by specific bacterial fragments. The possibility of hyper-activation of PRRs in inflammatory conditions suggests further function of endothelial cells as a second barrier against antigens (91).

Expansion of the microvasculature in inflamed intestinal tissue stimulates the recruitment of pro-inflammatory mediators, thus further amplifying inflammation and tissue damage.

III. PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINES IN IBD

Under normal conditions, inflammatory reactions within the intestinal mucosa are quantitatively and temporally controlled by a delicate balance between pro-inflammatory (TNF α , IFN γ , IL-1, IL-6, IL-8, IL-17, IL-23) and anti-inflammatory (IL-4, IL-10, IL-11, TGF β) cytokines.

The main hypothesis on the development and progression of IBD is based on impairment of immune tolerance to the gut commensal microbiota, thought to be due to a genetic predisposition of the host, which leads to chronic intestinal inflammation and mucosal damage. The chronic inflammation leads to massive gut infiltration by granulocytes and macrophages, with different features in CD and UC, as depicted in Figures 2 and 3. These cells produce large amounts of pro-inflammatory cytokines, chemokines, and ROS/RNS intermediates, which the anti-inflammatory cytokines and antioxidant molecules are unable to counteract.

Important is the role played by immune cells, which also mediate inflammatory reactions through the expression and synthesis of different cytokines. In IBD patients, the inflamed mucosa shows active infiltration of CD4⁺ T Th lymphocytes, which are responsible for maintaining chronic inflammation. In general, naïve CD4⁺ Th cells proliferate and differentiate into different subsets upon stimulation by specific cytokines: three different Th, i.e. Th1, Th2, and Th17, and a subpopulation of regulatory T lymphocytes (Tregs) are formed. Each T cell subset secretes specific cytokines able to differentially modulate inflammatory reactions.

III A. Cytokines produced by T helper cells

Lymphocytes Th1 are the main mediators of type1 immunity. They act against intracellular pathogens by means of intense phagocyte activity and produce IL-2, IFN γ , and TNF α ; the same cytokines amplify this response by inducing macrophages to release further amounts of TNF α , IL-6, and IL-1 β . Th1 cells and related cytokines are predominant in active CD. The histopathological features of CD are shown in Figure 4. The expressions of IL-1 β , IL-6 and TNF α are increased in the affected mucosa of patients with active IBD. Conversely, the same patients showed decreased levels of IL-2 and IFN γ in the peripheral blood cells (209).

Among the various cytokines produced during the Th1 response, the overexpression of TNF α undoubtedly plays a key role in the induction of intestinal damage, especially in CD patients. This molecule can trigger pro-inflammatory and survival pathways, as well as the apoptotic process. Its effect probably depends on the amount and time of cell exposure to the cytokine, the type of receptor

that binds TNF α , and the sequence of events that take place upon ligand binding. Regulation of NF- κ B activity appears to be fundamental in determining the specific cellular response to TNF α (211). Based on the extensive research that has been done into the biological activity of this molecule, the use of TNF α blockers in treating CD has recently generated considerable interest.

Cytokines produced by Th2 cells are selectively increased in the inflamed mucosa of UC. These cells synthesize IL-4, IL-5, IL-10, and IL-13, and are required for humoral response (185). Th2 lymphocytes support the synthesis of all types of immunoglobulins, by secreting IL-4 and IL-5, and inhibit Th1-activated macrophages by secreting IL-4, IL-10 and IL-13. IL-4 and IL-10 are considered anti-inflammatory cytokines. IL-4 secretion by dendritic cells is also a fundamental up-stream effector of Th2 differentiation. Conversely, IL-13 is considered a pro-inflammatory molecule mostly implicated in allergic inflammation. Mononuclear cells from intestinal lamina propria of UC patients show high levels of Th2-related IL-13 (92).

The function of specific lymphocytes is more complicated. For example, both Th1 and Th2 cells can increase transcription of *MUC2*, through NF- κ B activation. Th2 cells utilize IL-4 and IL-13, which mediate NF- κ B-dependent *MUC2* transcription through MAPK signaling. On the other hand, Th1 cells up-regulate NF- κ B-dependent *MUC2* transcription through the TNF α -phosphatidylinositol 3 kinase (PI3K)/Akt cell pathway (124).

Lymphocytes Th17 secrete pro-inflammatory cytokines IL-17, IL-22, TNF α and IL-6, and have been identified as potent inducers of tissue inflammation because they recruit neutrophils and macrophages when Th1-type and Th2-type immunity fails to clear pathogens (156). Essential for maintaining Th17 response is the cytokine IL-23, and is characteristically associated with Th17 cell lineage differentiation and expansion. Furthermore, IL-23 counteracts anti-inflammatory pathways, by inhibiting the generation of inducible T regulatory cells, and reducing IL-10 secretion by other CD4⁺ T cells (175).

IIIB. Cytokines produced by T regulatory cells

Treg cell subsets are activated by the two anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF β), mainly those produced by dendritic cells (46). They are characterized by the expression of the transcription factor forkhead box protein 3 (FoxP3), and show the so-called CD4⁺CD25(high) phenotype in humans. They are thus also termed CD4⁺CD25(high)FoxP3⁺ cells. During the active phase of the disease, both CD and UC patients showed a decreased number of peripheral suppressive Tregs, whereas an expansion of the Treg population has been found in mucosal lymphoid tissues of these patients (20, 295).

Tregs may indirectly control the production of pro-inflammatory TNF α , through the T-box transcription factor T-bet. T-bet deficient mice developing severe colitis showed a selective increase of TNF α production by colon dendritic cells. This finding suggests that colonic T-bet deficiency in the dendritic cells may confer genetic susceptibility to aberrant TNF α -related inflammatory response, by triggering strong apoptotic processes in the intestinal epithelium, which becomes more sensitive to pathogenic microbiota (79).

IL-10 and TGF β have long been known as the two main cytokines involved in the negative regulation of inflammation and immune response, thus contributing to the maintenance of gut homeostasis.

The protective role of IL-10 in IBD had already been demonstrated in *IL-10* knock-out mouse model of colitis, which required gut microbial intervention to develop inflammation. This gave rise to the idea that IL-10 could be involved in restricting the mucosal immune response to the enteric flora (138). IL-10 inhibits pro-inflammatory cytokine expression and nitric oxide (NO) generation in macrophages. It has been observed that IL-10 activates different cell signaling pathways in order to exert its anti-inflammatory action. In particular, it promotes proliferation in selected immune cells through JAK/STAT3 or PI3K/Akt pathways. IL-10 can also inhibit TNF α production and hemeoxygenase-1 induction in macrophages through the activation of p38 MAPK pathway (201). Impairment of IL-10 signaling has been reported in a pediatric group of IBD patients with granuloma-positive colitis (20). These findings suggest IL-10 is a potential therapeutic target for IBD, although

no apparent beneficial effect of IL-10 therapy has yet been observed in a number of human clinical trials, probably due to IL-10 instability and its short half-life (114).

The TGF β cytokine family is recognized as a key regulator of cell proliferation, differentiation and apoptosis in the intestinal mucosa, as well as of the control of immunological self-tolerance, inducing Treg activation by up-regulating FoxP3; high levels of the TGF β 1 isoform have been found to inhibit induction of Th17 cells (297). TGF β 1 is very probably implicated in organ fibrosis, and may be overexpressed by CD stricture fibroblasts. Tissue levels of this cytokine are transiently diminished in the early phases of IBD, whereas they increase at later disease stages (33,110). It has been hypothesized that the TGF β 1 cell signaling pathway is defective in IBD: up-regulation of Smad7, the intracellular inhibitory molecule of the TGF β 1 signal, followed by a block of phosphorylation of the principal transduction molecule Smad3, have been reported to occur in murine IBD models (173). Finally, TGF β 1 has long been recognized to possess marked pro-angiogenic activity, so that the pleiotropic action of this cytokine appears to play a primary role in the chronicization of IBD (Figure 5).

IV. THE MAIN GENETIC POLYMORPHISMS ASSOCIATED WITH IBD

Although the precise etiology of IBD remains unclear, genome-wide association studies have identified more than 100 loci that are significantly associated with IBD, and that confer susceptibility to this disease. However, the contribution of genetic factors to IBD is complex: several putative susceptibility genes are involved in the pathogenesis of IBD and may interact with one another (271). Approximately 70 loci are associated with CD and 50 with UC, spanning pathways involved in the host defence (17, 76, 103, 236). Although CD and UC share some susceptibility genes, implicated in various different intestinal functions, they also have distinct association patterns. In this connection, CD is associated with *NOD2* and with genes that regulate autophagy, whereas the predominant association in UC is with the human leukocyte antigen (HLA) class II genes (165), as well as with genes involved in the epithelial defense function, such as *cadherin*, *laminin β 1*, and *MUC19* (6, 165).

The first and most significant genetic variant reported to be associated with an increased susceptibility to CD, but not to UC, is *CARD15/NOD2*; this can lead to defective mucosal recognition of bacteria, with persistent activation of NF- κ B and inflammatory reactions (191). However, advances in molecular genetics indicate that mutations in *NOD2* alone are not sufficient to cause CD. The association of *CARD15/NOD2* variants with genes encoding for autophagy process, in particular with autophagy-related protein (*ATG16L*), has been widely reported (17). Other essential genes for autophagy, i.e. *immunity-related guanosine triphosphatase M (IRGM)* (164) or *leucine-rich repeat kinase 2 (LRRK2)* (267) have also been associated to CD.

Among HLA class II genes, which regulate interactions between host cells and pathogens, *DRB1*0103* and *DRB1*1502* have been reported to confer an increased risk of UC, whereas *DRB1*0410* and *DQB*0401* confer a risk for CD. Increased risk of UC has also been associated to *B52 HLA* class I gene, whereas *Cw8* and *B21* confer a risk for CD (68).

Additional multiple variants have been observed in CD and/or UC, associated with the potential induction of defective innate immune pathways. A mutation of the *D299G/TLR4* gene appears to be associated with some Caucasian CD and UC populations (75). A common variant in the *NLRP3* region gene has been found to be closely associated with the risk of CD (273).

IL-23R polymorphisms have been associated with CD (152). *IL-23R* and *IL-17A* variants have been linked to both UC and CD in part of the Korean population, and a significant polymorphism in the promoter region of *IL-17A* has been identified (123). Other susceptibility genes involved in the IL-23 pathway, such as *IL-12B*, *JAK2*, and *STAT3*, have also been associated with CD (280). *IL-22* has recently been named as a potential susceptibility gene in UC (236), and polymorphisms of *IL-10* receptor 2, which is shared by both IL-10 and IL-22, has been found in children affected by early-onset enterocolitis (82).

Different IBD-associated risk loci are reported to be shared by CD and UC; however, findings from human studies are still insufficient to draw any conclusions.

V. REDOX IMBALANCE IN IBD

The application of modern molecular biology to the investigation of IBD pathogenesis, employing murine experimental models that mimic the disease's pathophysiology and biopsies of patients' affected mucosa, has provided new insights into an old problem: the actual role of redox imbalance in disease initiation and progression.

V A. Antioxidant defenses in IBD

1) Alteration of redox couples in the IBD-affected intestinal tract

The redox state of the intestinal epithelium plays a fundamental role in maintaining cell integrity and function, modulating signal transduction, absorbing nutrients, and interacting with luminal microflora. Key players in the intestinal redox biology appear to be three redox couples, namely reduced glutathione/glutathione disulfide (GSH/GSSG), cysteine/cystine (Cys/CySS) and reduced and oxidized thioredoxin (Trx/TrxSS). A recent review comprehensively analyzed their physiological role and function (42).

The antioxidant system, represented by glutathione and glutathione-related molecules and enzymes, was probably the first to be investigated in depth in IBD patients and in relevant experimental models. In a cohort of 33 adult patients with active CD, Dröge's group was the first to show substantial changes in GSH levels in the intestinal mucosa, namely of the ileum tract (234): whereas they found GSH and GSSG levels to be within the control range in red blood cells and circulating mononuclear cells, the GSH content of the inflamed ileum mucosa was markedly reduced, by about 40%. Very interestingly, the decreased GSH levels were not limited to the inflamed intestinal mucosa, but were also observed in bioptic samples of non-inflamed mucosa from the same patients (about 25% decrease). Moreover, GSSG levels were found to be very significantly increased only in inflamed areas, with a consequent net imbalance of the GSH/GSSG ratio. The same group found that γ -glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis, was markedly inactivated, again showing an approximate 50% decrease in inflamed mucosa and 25% decrease in non-inflamed mucosa. Similar findings were obtained for γ -glutamyltransferase, an essential enzyme for GSH re-synthesis, which exhibited marked inactivation, with a consequent net GSH decrease,

especially in the inflamed mucosa (234). A net deficit in GSH synthesis was then demonstrated in the ileal mucosa of CD patients, which was at least in part independent of inflammation, and thus not a mere consequence of it.

GSH depletion, most likely a crucial event in intestinal damage during IBD, has been fully confirmed by other research, both in patients and in experimental animals. In a smaller group of adult patients with active CD (n=18), several of whom were also malnourished, the amount of GSH recovered from the intestinal mucosa appeared to be diminished by about 70% versus controls. But again, a low concentration of GSH was observed both in inflamed and in non-inflamed mucosal samples. In addition, malnutrition significantly worsened the tripeptide deficiency (168).

A decrease of GSH in the intestinal mucosa of IBD patients thus appears to be an early event in the natural history of the disease, not simply a consequence of overconsumption of antioxidants, due to the associated condition of oxidative stress. With the progression of the disease, impaired intestinal absorption of nutrients may accelerate and worsen the evolution of the disease process in many patients. This has been consistently confirmed by experimental attempts to replenish the GSH content of the intestinal mucosa in animal IBD models. In the case of TNBS/ethanol-induced colitis in rats, administration of the GSH precursor N-acetylcysteine (NAC) doubled the tripeptide pool in the intestinal mucosa, and reduced the extension of gut mucosa damage by 60-70% (8). A marked reduction of intestinal mucosa damage was also obtained in the TNBS model, both by supplementing the rats with a single dose of GSH before colitis induction (effect on disease onset) and by giving them repeated daily amounts of the tripeptide after colitis induction (effect on disease progression). In the first condition, GSH supplementation partly but significantly prevented lipid peroxidation and tissue damage; in the chronic model, daily GSH intraperitoneal injection completely restored the GSH loss and afforded good control of mucosal injury (158). In DSS-induced colitis in mice gut mucosal levels of GSH were improved by NAC treatment, and histological damage of the colonic mucosa was partially prevented (294).

With regard to the second redox couple mentioned above, i.e. Cys/CySS, again Dröge's group reported a moderate decrease in plasma levels of the two amino acids, in both CD and UC patients,

before surgical removal of the inflamed tract, with a rapid return to normal plasma values after surgery. The latter finding appears to indicate that the decrease of Cys and CySS plasma levels observed in IBD patients was secondary to inflammation (234). IEC take up Cys, either as such from the diet, or through trans-sulfuration of dietary methionine. A significant proportion of the Cys absorbed by the gut leads to the formation of GSH. In addition, Cys is present in the functional motif of the major antioxidant enzymes, including Trx, and is a component of the regulatory and/or catalytic sites of a number of transcription factors, kinases, and phosphatases. Together with the GSH-GSSG couple, the Cys/CySS equilibrium regulates cell survival and function (34).

Changes to another important redox couple, namely the reduced and the oxidized forms of Trx, have also been considered in the pathogenesis of IBD. Trx are small proteins containing a redox-active disulfide/dithiol group within the conserved active site sequence Cys32-Gly-Pro-Cys35. Of the two main Trx found in mammals, Trx-1 has been studied more widely. It catalyzes the reversible reduction of disulfide bonds, deriving from oxidation of critical Cys residues in proteins. Perturbation of the Trx system in the intestinal mucosa of CD and UC patients has not as such yet been reported, and is thus still a matter of speculation. However, indirect indications of its occurrence in IBD come from recent findings of a marked increase of Trx-1 in the blood serum of CD and UC patients in the active disease phase, versus controls and versus IBD patients with inactive disease, who exhibited values consistently within the normal range (252). This group speculated that such a marked rise in serum Trx was evidence of a stimulated antioxidant host response, due to oxidative stress and inflammation in the gut mucosa, but the study did not provide direct proof of this. However, in a DSS mouse model of experimental colitis, they clearly demonstrated that preventive or therapeutic administration of recombinant human Trx to the affected animals resulted in a net amelioration of the disease state, and limited mucosal damage (252).

Two findings support an increased level of Trx in the inflamed gut mucosa of IBD patients: i) expression of this protein can be induced by oxidative stress of moderate extent, an event already described for small intestine epithelial cells several years ago (94) and ii) the recent report of down-regulated expression of the Trx negative regulator, known as thioredoxin interacting protein (Trxip),

within the inflamed mucosa of UC patients (251). Conversely, however, expression of Trxip has recently been reported to be significantly up-regulated by oxidative stress (121), but this controversial finding was in mice undergoing transient focal cerebral ischemia.

2) Impairment of enzymatic antioxidant defense in IBD

IBD is characterized by marked but differing alterations of various enzymes involved in the oxidant/antioxidant homeostasis of IEC and mucosa. In terms of pathogenesis, important changes have been observed to occur in superoxide dismutase (SOD) and catalase (CAT) activities and/or levels in the intestinal mucosa of IBD patients. In a cohort of 19 CD and 15 UC patients, MnSOD level in inflamed gut mucosa increased by approximately 2.5 fold versus control mucosa, rather similarly in the two diseases. Notably, also in the non-inflamed IBD mucosa, MnSOD level showed an average increase of 56%. However, enzyme activity did not differ significantly versus controls. In the same group of patients, Cu/ZnSOD tissue levels increased 1.5 fold in the non-inflamed IBD intestinal mucosa, but not in inflamed areas. Extracellular SOD levels tended to decrease both in inflamed and in non-inflamed IBD mucosa (132).

Another study on lymphocytes, granulocytes and monocytes from peripheral blood of 20-25 CD patients, both at disease onset and after remission, confirmed an increase in total SOD activity in the blood during the early clinical phases of the disease, with a return to control values after remission. Consistently, hydrogen peroxide H_2O_2 steady-state concentration in all three types of cells increased during the active phase of CD, returning within the control range in the inactive phase (21, 101). All these findings indicate that total SOD activity is up-regulated in IBD patients as a reaction to oxidative stress and inflammation. However, the observed increase of MnSOD and Cu/ZnSOD protein levels in non-inflamed areas of CD and UC intestinal mucosa would suggest a possible increase in the generation of H_2O_2 , i.e. the product of SOD activity, also during disease remission, and thus regardless of ongoing inflammatory reactions.

In connection with the observed up-regulation of SOD in IBD, it is important to pinpoint both disease activity and levels of CAT, i.e. the enzyme converting $2H_2O_2$ into $2H_2O$ and O_2 . In the cohort

of CD patients studied by Beltran and colleagues, CAT activity measured in lymphocytes from patients with active disease was half that of controls. Moreover, this reduced enzyme activity persisted in the lymphocytes of the same patients after disease remission, and was also confirmed in CD patients with inactive disease (21, 101). The overall finding of an inhibited CAT activity in the lymphocytes of CD patients matches the evidence of reduced CAT activity in the erythrocytes of CD or UC patients in the active phase. On the other hand, in contrast to that observed in the lymphocytes, CAT activity showed normal values in the red blood cells of patients with inactive IBD (136).

From the available literature, which indicates an increase in SOD and a decrease in CAT, at least in immunocompetent cells and erythrocytes from IBD patients, changes that are not strictly dependent on the inflammatory state of the disease, it might be hypothesized that inflammation-independent changes of antioxidant enzymes may sustain an increased steady-state concentration of H_2O_2 , and consequently of ROS, during remission of the disease process, and possibly also before its first clinical expression.

Again concerning antioxidant enzymatic defenses, the observed decrease of GSH, an important intracellular antioxidant but also the substrate for a large family of selenium-containing enzymes with peroxidase activity (the glutathione peroxidases - Gpxs), might be crucial in the modulation of Gpxs' role in counteracting oxidative damage, in CD and in UC. Gpx-1, a ubiquitous enzyme, and Gpx-2, which is gastrointestinal, recognize H_2O_2 as their main substrate, whereas Gpx-4, which is ubiquitous, shows much more affinity for lipid hydroperoxides. Of note, in the *ex vivo* evaluation of Gpx activity, the substrate GSH was added externally to perform the analytical test, meaning that any deficit in the mucosa was not taken into account. It would be of interest to know the actual levels of this enzyme within the IBD mucosa, during inactive and active disease phases. Despite inconsistency among different reports on Gpx activity in the peripheral blood of IBD patients (213), the conclusions drawn by a further study by Kruidenier and colleagues appear to be informative and reliable (132). This study measured activity and levels of Gpx and CAT, in the same colon biopsies that had been employed to analyze SOD isoenzymes. When total Gpx and CAT activity was evaluated in the whole IBD mucosa, both enzymes were up-regulated. In particular, in the lamina

propria of inflamed areas, an increased amount of CAT was detectable in phagocytes, which also express myeloperoxidase (MPO); on the contrary, the CAT level within the epithelium was very low, only a few cells expressing Gpx. These findings further point to hampered removal of H_2O_2 in the IBD colonic mucosa, particularly at the level of the epithelium. A more recent analysis of bioptic fragments of damaged mucosa from subjects affected by either UC or CD gave consistent results: the intestinal isoform of Gpx-2 was found to be up-regulated; conversely, aquaporin 8, one of the proteins forming pores in biomembranes and allowing the diffusion of H_2O_2 , was found to be down-regulated (256).

3) Antioxidant vitamins and antioxidant-related trace metals in IBD mucosa

A number of reports are available on the level of β -carotene and vitamins A, C and E, in the plasma/serum of patients with CD or UC (213, 104). The large majority of these studies found a deficient blood content of antioxidant vitamins in IBD. In this connection, amelioration of the disease severity in patients supplemented with vitamin E, with or without vitamins A and C, has been reported (207, 2). Moreover, most reports relate to IBD in the active clinical phase, and do not discriminate between reduced dietary supply, due to gut malabsorption, and sacrificial consumption to counteract oxidative stress. Undoubtedly, both conditions may severely affect the antioxidant vitamin status of IBD patients, especially during activation of inflammatory and oxidative damage of the intestinal mucosa. For all these reasons, it would be of great interest to measure the various antioxidants in the blood of IBD individuals, during inactive and during remitted disease.

In a cohort of 54 consecutive patients with CD, in remission for more than three months, Filippi and colleagues found that a large proportion of cases were characterized by low plasma levels of vitamin C (85% of patients); plasma levels of β -carotene and vitamin E were within the normal range in about one quarter of patients, while low levels of vitamin A were detected only in 4-5% of cases (70).

More recently, antioxidant vitamins and pre-vitamins, as well as antioxidant-related trace metals, were measured comprehensively in a multicenter study on 132 IBD (CD and UC) patients in a

quiescent disease state, and in 35 patients with clinical symptoms. Of note, most of the patients with quiescent disease showed normal nutritional status, whereas the nutritional status of those with active disease was deficient. Versus healthy controls (n=45), quiescent IBD patients exhibited significantly lower plasma levels of vitamins E and C, as well as of several carotenoids, including β -carotene, lutein and lycopene. When quiescent versus active IBD patients were compared, plasma vitamin E levels appeared similar in the two groups, while vitamin C and the above carotenoids showed a further decrease during the active process (93). With regard to trace elements, plasma copper increased and selenium decreased in the active disease group. The latter finding confirms all previous related reports that consistently showed low concentrations of this mineral in the plasma of UC and CD patients (213). It may, also indirectly explain the repeated finding of impaired seleno-enzyme Gpx in IBD-affected intestinal epithelium (132).

More importantly, the significant deficit in plasma antioxidant vitamins and provitamins, in patients with quiescent IBD disease and normal nutritional status, did not appear to depend on either malabsorption or inflammation, but rather on specific dietary limitations (low intake of fruits and vegetables) undertaken by the patients (93).

4) Dietary polyphenols and inflammation of the gut mucosa

Besides tocopherols and tocotrienols, i.e. antioxidants bearing one phenolic ring, there are many other plant-derived phenols, with much stronger antioxidant power as tested *in vitro*, possibly due to their multiple phenolic rings; collectively these are termed polyphenols. Most of the compounds belonging to this large family are strong electron donors and metal chelators, thus able to quench or terminate free radicals chain reactions (149). Among the subclasses of polyphenols, those of high biomedical interest are the stilbenes (e.g. resveratrol), and the flavonoids. The flavonoids are further subdivided into anthocyanidins (e.g. malvidin), flavonols (e.g. quercetin), monomeric flavanols (e.g. catechin), polymeric flavanols (e.g. procyanidins), flavanones (e.g. naringenin), flavons (e.g. luteinin), and isoflavones (e.g. genistein, daidzein) (73, 24). In general, dietary polyphenols display poor *in vivo* bioavailability, mainly due to their scarce absorption and rapid metabolism in the gut. For this reason,

it is neither worth-while nor reliable to measure their concentrations in the peripheral blood; however, an increasing bulk of literature re-examines these compounds in terms of their possible contribution to the intestinal redox environment and strong anti-inflammatory potential. In IBD, the integrity of the intestinal mucosa and its barrier function are severely impaired, so that compounds such as polyphenols might more easily interact with attacking agents, and with inflammatory or immune cells.

Based on epidemiological studies suggesting an important role of dietary flavonoids in helping to prevent disease processes sustained by inflammatory reactions (63, 56), a number of investigations have used in *in vitro* and *in vivo* experimental models to assess the effect of chronic consumption of polyphenols on soluble adhesion molecules and inflammatory cytokines. Using IEC lines derived from human colon cancer, most of the polyphenols examined, proved to significantly quench, at various levels, NF- κ B activation and nuclear translocation pathway, and also to down-regulate the synthesis and secretion of inflammatory cytokines including IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1). However, genistein and a few other flavonoids gave controversial results, and resveratrol even appeared to up-regulate IL-8 secretion by colonic cells (217). The findings of a large number of studies on animals with experimental colitis, concerning the anti-inflammatory and overall protective action of polyphenols, are more consistent. Almost all such reports showed that several polyphenols, given as single compounds or as a mixture (plant polyphenolic extracts), can significantly attenuate gut mucosal inflammation and related damage. Among the plant polyphenolic extracts, the most widely investigated ones were those deriving from green tea (217).

Flavanol epigallocatechin gallate, a major component of green tea, was demonstrated to markedly down-regulate the expression of IL-17 and TNF α in a human T cell line (Kit 225) overproducing those cytokines (55). The same flavonoid had previously been shown to inhibit TNF α -induced increase of IL-8 and prostaglandin (PG) E₂ production in human colonadenocarcinoma cell lines (204).

Recently, using the database of the U.S. Nurses' Health Study, which includes more than 100,000 apparently healthy female nurses in the USA, Landberg and colleagues analyzed any possible relationship between dietary intake of flavonoids and plasma biomarkers of inflammation and

endothelial dysfunction. An inverse association was found between total intake of flavonoids and plasma levels of the pro-inflammatory cytokine IL-18, and between flavonols (catechins) and vascular adhesion molecule-1 (VCAM-1) (142).

Table 1 reports the main alterations of antioxidant defenses observed in human inflammatory bowel diseases.

V B. Oxidative insult in IBD

A significant mass of data now points to a preponderance of ROS and RNS being the antioxidant defense affected in the inflamed areas of the gut of IBD. Quite recently, experimental and human studies on the steady-state of ROS and antioxidants in the inflamed colon mucosa have been nicely put together and analyzed (213). IBD is a classic chronic disease process in which inflammatory cells, cytokines, chemokines, and intestinal flora interact, until they induce a net imbalance of the redox equilibrium of colonic mucosa, with the consequent development of an “oxy-radical overload” (181, 202, 269). ROS levels have been correlated to the clinical and endoscopic assessment of IBD severity (134). Of note, RNS and ROS have been demonstrated to up-regulate a number of different genes involved in adaptive and innate immune responses in the gastrointestinal tract.

1) RNS in IBD progression

Nitric oxide steady-state levels in the bowel mucosa during the development of inflammatory diseases may be significantly altered because of marked changes in both the synthesis and the disposition of this reactive chemical species. Enhanced recruitment of phagocytic cells, followed by the increased inducible nitric oxide synthase (iNOS) concentration, which occur in IBD affected intestinal mucosa, are accompanied by peculiar modifications of the local endothelium, expressing both iNOS and endothelial nitric oxide synthase (eNOS).

The marked expansion of the microvasculature in the chronically-inflamed intestinal mucosa magnifies the harmful effects to the altered endothelium in IBD. The affected intestinal endothelium

generally shows a diminished amount of eNOS versus its normal counterpart (72). Markedly reduced NO-dependent vasodilation was demonstrated by *in vitro* videomicroscopy in human submucosal arterioles that had been dissected from IBD intestinal specimens (88). Moreover, the lack of expression of eNOS in the intestinal mucosa, as experimentally achieved in *eNOS*^{-/-} mice, led to a marked reduction of plasma nitrite versus wild-type animals and, in addition, severe exacerbation of colitis induced by DSS (221).

Another very important source of NO in intestinal endothelial cells is iNOS, which, unlike the inducible isoform present in leukocytes, appears to play a physiological role protecting against leukocyte adhesion (28). In agreement with this evidence, DSS-induced inflammation of colonic mucosa was found to be more severe in *iNOS*^{-/-} mice than in DSS-treated wild-type mice (131). As in the case of eNOS, levels of iNOS were also found to be consistently decreased in intestinal endothelial cells isolated from surgical samples of IBD mucosa, from both CD and UC patients (28). Importantly, despite the fact that both the inducible and the constitutive forms of NO synthases present in normal intestinal microvasculature are markedly reduced in IBD, the overall amount of NO in this chronically-inflamed gut mucosa has clearly been shown to be significantly increased due to tissue invasion by inflammatory cells, which increase local concentrations of iNOS (128, 131). That excessive generation of NO, mainly sustained by iNOS activity, is implicated in the pathogenesis of IBD as supported by the finding that in mice lacking STAT-6 challenged with DSS the resulting colitis is much more severe. Significantly, *STAT-6*^{-/-} mice showed increased levels of iNOS, suggesting a regulatory role of STAT-6 on iNOS activity (62). Moreover, continuous cytokine exposure during chronic inflammation, and excessive consumption of nitrite- and nitrate-rich foods, may induce DNA deamination and cause strand breakage and mutations (225). Peroxynitrite (ONOO⁻) produced via iNOS during chronic inflammation can cause nitrative DNA damage by forming 8-nitroguanine, in both animals and humans with inflammatory diseases, included intestinal diseases (113).

Taking all available findings together, it might be concluded that, during the development of IBD, a net derangement of NO synthesis by the endothelial cells occurs, which is apparently directly

related to the severity/exacerbation of the inflammatory process. The physiological protective role of endothelial NO is lost, overwhelmed by an excess of NO of leukocyte derivation; this NO, in addition, shows localization and likely diffusion rates that differ significantly from those of the constitutive chemical species (279).

2) ROS in IBD progression

The primary activity of ROS in defense against invading microbes is the well-known respiratory burst induced in phagocytic cells. In particular, stimulated macrophages generate high concentrations of ONOO^- and H_2O_2 , acting as bactericidal agents. ROS comprise many species, i.e. superoxide (O_2^-), hydroxyl radical (HO^\cdot) and H_2O_2 . Various studies have demonstrated that ROS are implicated in determining mucosal injury in UC (206). Gene expression profiles in the three different mouse models of colitis revealed alterations in the expression of important genes involved in the regulation of H_2O_2 production in the intestinal tissue (256). Furthermore, the mucosal barrier dysfunction has been related to disorganization of the cytoskeleton in epithelial cells, due to excessive ROS generation (14).

An excess of ROS causes massive DNA damage to accumulate. Reaction of the hydroxyl radical at the C-8 position of deoxyguanosine induces formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is considered a marker of oxidative DNA damage in biological systems (83). Oxidative DNA lesions are relevant to mutagenesis, producing several base modifications such as G:C to T:A transversions, which have been associated to *TP53* mutations in UC (100). The colonic mucosa concentration of 8-OHdG has been found to be doubled in rats treated with DSS. The role of inflammation in inducing DNA oxidative damage, in terms of 8-OHdG production, has been verified in colonocytes by using the cyclooxygenase (COX)-2 inhibitor nimesulide (253, 254). It is widely thought that chronic inflammation is actively involved in intestinal tumorigenesis via the induction of DNA mutations by ROS (58, 67, 166).

An important source of oxidative DNA damage is the mitochondria, because of the central role they play in energy oxidative cell metabolism. Mitochondrial DNA is particularly exposed to

ROS attack, due to its localization, close to the intracellular electron transport chain in the inner mitochondrial membrane, and to the absence of protective histones. Polymerase chain reaction analyses to detect large deletions in the mitochondrial DNA, and 8-OHdG tissue concentrations, were measured in mucosal specimens from patients affected by UC. The increased gene instability of mitochondrial DNA in UC patients has been related to the high development rate of colorectal cancer (188).

However, owing to their short half-life and high reactivity, direct detection of ROS *in vivo* is difficult. Oxidative epithelial-cell injury has been indirectly evaluated in the mucosa of IBD patients, in whom the production of oxidants was excessive if compared to their antioxidant defenses (134). The assessment of oxidative and/or nitrative modifications of proteins has long been one of the few methods available to detect ROS/RNS generation *in vivo*. Inflamed mucosa of UC patients with active disease shows increased cytoskeletal protein oxidation and nitration (116). Direct evidence of *in vivo* ROS production in IBD has been provided using chemiluminescence methods (192, 247) or fluorescent probes (see Figure 6) detecting $O_2^{\cdot-}$, H_2O_2 , and NO in lymphocytes, monocytes, and granulocytes isolated from the peripheral blood of both CD and UC patients (21).

Recent studies, aimed at evaluating the therapeutic effect of probiotics, have demonstrated abnormal intestinal bacterial colonization in IBD, which often causes damage by inducing exaggerated oxidative reactions. Certain bacteria, such as *Bacteroides*, *Clostridium* and *Enterococcus*, can be considered detrimental, whereas *Bifidobacterium* and *Lactobacillus* may be beneficial (65). *Enterococcus faecalis* has been implicated in tissue damage in IBD because of its ability to induce high concentrations of H_2O_2 and of inflammatory cytokines, such as TNF α and IL-12p70 (161). ROS production is critical for efficient autophagic targeting of *Salmonella enterica* serovar Typhimurium in phagocytes (98). Conversely, *Lactobacillus* strains may ameliorate IBD severity, by activating antioxidant enzymes and anti-inflammatory cytokines, both in experimental animal models and in children (37, 153).

The importance of ROS has been stressed in terms of their bactericidal effect, as regulators of molecules involved in the recognition and processing of intestinal pathogens. ROS are essential for

autophagosomal degradation of proteins: in particular, H_2O_2 specifically inactivates ATG4, most likely activating PI3K signaling, thereby promoting ATG8 lipidation, and resulting in enhanced autophagy (223). Conversely, another *in vitro* study has shown that ATG16L1 dysfunction, which has been observed in CD patients, results in an overproduction of ROS, which diverts cells away from autophagy, to activating NLRP3-related IL-1 β production (219).

ROS generation is closely related to TLR and NLR recognition aimed at killing the pathogen directly. ROS are reported to be involved in the NF- κ B pathway activation mediated by TLR4 in neutrophils (12). Autophagy induced by the LPS is mediated by TLR4-induced signaling. Generation of ROS with consequent recruitment of autophagy light chain peptide LC3 to phagosomes during phagocytosis of TLRs has been shown in the murine macrophage Raw 264.7 cell line (289). Various stressors, such as asbestos and silicas, activated NLRP3 to secrete IL-1 β in macrophages through ROS generation mediated by ATP. ROS can activate the PI3K transduction pathway, which in turn is required to activate pro-caspase-1 and to secrete pro-inflammatory IL-1 and IL-18 (50, 61). ROS may either directly activate inflammasomes, or indirectly interact with other molecules able to modulate inflammasome activity (162).

3) The role of lipid oxidation end-products in IBD

Most studies determined the presence of a condition of oxidative stress in experimental colitis and/or human IBD in terms of increased lipid oxidation products, measuring thiobarbituric acid reacting substances or aldehydic end-products, such as 4-hydroxynonenal (HNE) and malondialdehyde (213). Dietary lipids, in particular ω 6-polyunsaturated fatty acids (PUFAs) and their oxidative modifications are undoubtedly crucial in promoting chronic inflammation: they provide the main substrates for PGs and leukotrienes (LTs) formation through COXs and lipoxygenases (LOXs) activity and, for reactive aldehyde production, by non-enzymatic breakdown.

As far as COX activity is concerned, phagocytes of the lamina propria produce high levels of ω 6-arachidonic acid (AA) metabolites through inducible COX-2. The eicosanoids PGE2 and LTB4

may activate Th cells to synthesize either pro- or anti-inflammatory mediators of the response to dietary antigens, by selectively triggering different signal transduction pathways (87).

A large body of literature is now available on cell-damaging effects of aldehydes deriving from non-enzymatic degradation of ω 6-PUFA; some of these carbonyl derivatives appear likely to be involved in chronic inflammatory processes characterized by excessive fibrogenesis, especially in atherosclerosis and neuronal diseases (184).

The aldehyde HNE is considered a crucial molecule in cell signaling activation, because of its strong reactivity with biomolecules containing amino and thiol groups. High concentrations of HNE exert necrogenic and genotoxic effects, whereas at low concentrations HNE appears to act as second messenger of free radical-mediated reactions. HNE appears to be involved in regulating a number of cell signals, especially in apoptosis- and inflammation-related signaling pathways. There is growing evidence of increased HNE tissue/blood levels in a wide variety of human diseases characterized by inflammation, pointing to the implication of this molecule in their pathogenesis (203).

Diet may influence tissue levels of HNE, which derives from arachidonic and linoleic ω 6-acids, which are often abundant especially in Western diets (246). However, HNE levels can be also affected by antioxidant intake, as well as by intestinal microbiota. HNE and its urinary metabolites have been found to be reduced as a response to improved plasma antioxidant concentrations in humans. In diabetic rats, dietary supplementation with the antioxidant α -lipoic acid reduced plasma levels of HNE and prevented the progression of diabetes mellitus complications, by inhibiting oxidative DNA damage of the peripheral lymphocytes. An *in vitro* model of dietary fiber fermentation decreased HNE-induced DNA damage and increased glutathione-S-transferase in IEC (13, 139, 222).

High levels of HNE have been consistently detected in the intestinal mucosa and in the plasma of CD patients (25, 26). An increase in etheno-modified DNA bases, generated by the reaction of DNA with HNE, has been found in inflamed pancreatic tissue of patients with chronic pancreatitis, and in the colon mucosa of IBD patients (178).

In male Sprague-Dawley rats, in which colitis was induced by LPS injection, a transient increase of HNE in colonic mucosa was shown to modify IgA molecules, thus resulting in a reduction

of bactericidal activity at the level of the intestinal epithelial layer (125). In other experimental models of colitis, induced by DSS or TNBS in C3H/HeN and C3H/HeJ mice, high levels of HNE and malonaldehyde, together with reduced antioxidant defenses (i.e. GSH content, SOD and CAT activities) have been detected in inflamed colon mucosa (144). The consistent evidence of HNE overproduction during inflammatory disease processes, together with HNE's strong reactivity with important functional biomolecules, point to this molecule being an effector of oxidative damage in IBD.

Besides lipid peroxidation-derived aldehydes, other lipid oxidation products might impact not only on IBD's progression and perpetuation, but also on its initiation. At least in the case of high dietary consumption of animal fats, several oxidation products of cholesterol, termed oxysterols, have recently been demonstrated to exert a strong pro-inflammatory effect on human colonic cells (163).

The pathogenetic role of dietary cholesterol and oxysterols in several inflammatory diseases, mainly atherosclerosis, has been established unequivocally (197, 241). Oxysterols belong to a family of 27-carbon cholesterol derivatives, which originate from the diet through non-enzymatic reactions, or endogenously through the activity of specific hydroxylases, cytochrome P450-dependent or -independent enzymes. Dietary oxysterols derive from cholesterol-rich food by autoxidation reactions, promoted either by incorrect food processing and storage, or by increased ROS steady-state levels (30). The addition of oxygen to the cholesterol molecule gives the resulting metabolite hydrophilic properties and much higher mobility than the parent compound within the cell membrane, with consequent changes in structure and function of the phospholipid bilayer. Dietary oxysterols are absorbed completely from the bowel, cleared from plasma into lipoproteins, and taken up by different tissues more rapidly than cholesterol (78). The principal dietary oxysterols formed upon non-enzymatic cholesterol oxidation are found in a mixture; they comprise 7 α -hydroxycholesterol, 7 β -hydroxycholesterol, 7-ketocholesterol, 5 α ,6 α -epoxycholesterol, and 5 β ,6 β -epoxycholesterol. Their concentrations are in the 10-100 μ M range in processed and/or stored cholesterol-rich foods (111).

Oxysterols perform important physiological activities that include sterol and bile acid biosynthesis and reverse cholesterol transport. However, the tissue accumulation of oxysterols has

been found to be potentially more pro-inflammatory and more toxic than cholesterol accumulation for different cell types, including IEC. The main cell pathways activated by oxysterols are summarized in Figure 7. A mixture of dietary oxysterols has recently been demonstrated to interfere with the homeostasis of the human digestive tract, promoting and sustaining inflammatory processes and inducing apoptosis of enterocyte-like CaCo-2 cells. In these cells oxysterols increased the expression of IL-1 β (see Figure 8), IL-6, IL-8, IL-23 and MCP-1, but also up-regulated mRNA levels of the recognition receptors TLR2 and TLR9. The NADPH oxidase isoform 1 (NOX1) appears to contribute to the pro-inflammatory and pro-apoptotic effects of oxysterols, since these events can be prevented by cell pre-treatment with the NADPH oxidase inhibitor diphenyleneiodonium (DPI). Among the oxysterols studied, 7 β -hydroxycholesterol and 7-ketocholesterol displayed the highest cytotoxicity. However, in food they are mixed with other oxysterols and do not elicit the same strong response, suggesting a partial competition with other oxysterols for the same cell functional sites (27, 163). Table 2 shows the main inflammatory molecules involved in IBD that are induced by lipid oxidation products.

In the mechanism by which ROS propagate inflammation a key role is certainly played by the activation of NF- κ B, which is involved in the regulation of several immune and inflammatory genes.

VI. REDOX STATE-RELATED TRANSCRIPTION FACTORS IN THE ETIOPATHOGENESIS OF IBD

VI A. Nuclear factor- κ B

The transcription factor NF- κ B is a major regulator of multiple important physiological processes. At the intestinal epithelium, NF- κ B is involved in the immune homeostasis of IEC, and in modulating intestinal barrier permeability (200). However, chronic NF- κ B activation may play a major role in exacerbating chronic inflammation of intestinal epithelium. This is supported by the aberrant NF- κ B activation consistently found in IBD (7, 215, 274). Furthermore, chronic NF- κ B activation and

the resulting inflammation are associated with a general risk for most cancer types (1), the link between IBD and colorectal cancer being paradigmatic (53, 66).

Transcription factor NF- κ B is composed of homodimers and heterodimers of the Rel/NF- κ B family of proteins (c-Rel, RelB, p65 [RelA], p50/p105 and p52/p100). NF- κ B normally resides in the cytosol, forming an inactive complex with the inhibitor of κ B (I κ B). Its activation via the canonical pathway involves I κ B kinase (IKK)-mediated phosphorylation of two I κ B α serine residues (S32 and S36). I κ B kinase consists of two regulatory subunits, IKK1 (IKK α) and IKK2 (IKK β), plus the NF- κ B essential modulator (NEMO or IKK γ) regulatory subunit. I κ B α phosphorylation triggers its subsequent ubiquitination and degradation by the proteasomes, and translocation of the active NF- κ B dimer to the nucleus. NF- κ B binds to DNA κ B sites and regulates the expression of many genes that can be grouped into those that modulate the immune and stress response, cell proliferation, and cell survival (288).

NF- κ B activation is a down-stream pathway in microbial recognition by IEC. Upon activation by different PAMPs, TLRs react with adaptor proteins MyD88 and TRIF, which in turn activate NF- κ B. Furthermore, recognition of the bacteria-derived peptoglycan by NLRs results in the recruitment of RIP2/RIP-like interacting caspase-like apoptosis regulatory protein kinase (RICK) and the subsequent activation of NF- κ B (3). Altered NF- κ B expression led to the dysregulation of antimicrobial peptides, which influenced the proliferation of pathogenic bacteria in the intestinal epithelium of *Drosophila melanogaster*, leading to the host's death (218).

NF- κ B is a redox-sensitive transcription factor. Increases in ROS tissue may partly explain the chronic activation of NF- κ B in IBD. ROS disrupt the redox interaction between the dynein light chain LC8 peptide and I κ B α , which prevents I κ B α phosphorylation by IKK (109). TNF α activates NF- κ B, which involves NADPH oxidase activation, increased ROS production, release of LC8, and I κ B α phosphorylation (107). In support of a role of oxidative stress in IBD-associated NF- κ B activation, phenyl-N-*tert*-butylnitron, an oxidant-trapping compound known to inhibit NF- κ B, ameliorates DSS-induced inflammation by inhibiting the expression of NF- κ B-regulated proteins, i.e. TNF α , IFN γ , and

iNOS (180). Consistently, this model of UC is associated with high levels of colonic mucosa oxidation and luminal nitrite/nitrate content (180).

Although the etiology of IBD is probably multifaceted, NF- κ B activation is a common finding in this disease. Administration of the NEMO-binding domain peptide, which blocks the association of NEMO with IKK β subunit, was found to inhibit IKK activation and reduce the severity of colon inflammation in different mouse models of colitis (232). High levels of p65 are found in macrophages, endothelial cells and lamina propria fibroblasts from IBD patients (80, 215). Hyper-activation of NF- κ B can cause an increased transcription of various genes that are central to IBD pathogenesis: a) pro-inflammatory cytokines: IL-1 β , IL-6, IL-8, IL-16 and TNF α (198), which are crucial in the IBD sustained cycle of intestinal barrier disruption by furthering the NF- κ B-dependent release of pro-inflammatory mediators (43, 255); b) genes regulating cell proliferation and survival: a NF- κ B-dependent increased expression of the pro-apoptotic p53 upregulated modulator of apoptosis (PUMA) protein contributes to colitis in humans and mice (208), while increased expression of certain NF- κ B-regulated genes may be involved in the proliferation and survival of transformed epithelial cells leading to tumorigenesis (43); c) genes regulating the permeability of the intestinal barrier: in this connection, TNF α induces the partial permeabilization of the intestinal barrier through NF- κ B-dependent up-regulation of myosin light chain kinase (292); d) metalloproteinases, which may degrade the extracellular matrix and mucosal cells (212); e) enzymes (e.g. COX-2 and iNOS), which by participating in the metabolism of ROS and RNS may contribute to NF- κ B activation and to the disruption of the barrier integrity (7) (Figure 9).

The relevance of NF- κ B dysregulation in IBD pathogenesis is supported by the evidence that drugs widely used in treating patients with IBD (i.e. sulfasalazine, steroids, TNF α antibodies) are capable of inhibiting NF- κ B (193, 265, 282). Furthermore, in support of a role of NF- κ B, induced IBD in mice was successfully treated with NF- κ B decoy oligonucleotides (69).

In summary, although NF- κ B has relevant physiological functions within the intestinal mucosa, its sustained activation accompanies the chronic inflammatory processes in IBD, and may

play a major role in both the etiopathogenesis of IBD and the IBD-associated higher risk for colorectal cancer development (193).

VI B. Nuclear factor-erythroid 2-related factor 2

In recent years, accelerating research has drawn attention to another redox-sensitive transcription factor, namely nuclear factor-erythroid 2-related factor (Nrf2), known to play a key role in the regulation of several genes coding for primary antioxidant and/or type 2 detoxifying enzymes (127, 172). Nrf2 may contribute to maintaining the homeostasis of the intestinal mucosa, by preventing and/or counteracting excess production of ROS within the intestinal epithelium and the lamina propria, as occurs in IBD. Further, Nrf2 appears to negatively modulate pro-inflammatory reactions occurring in the gut mucosa, through antioxidant and, possibly, non-antioxidant mechanisms. A number of *in vivo* experimental studies, most of which focused on lung inflammation and sepsis, have clarified the significant role of this transcription factor in regulating innate immunity and inflammatory processes (106, 266).

Direct evidence of the Nrf2-dependent negative regulation of gut inflammation was obtained in the DSS-induced mouse model of colitis using Nrf2 wild-type and knock-out (KO) animals. Colitis induced in *Nrf2*^{+/+} mice after 6-7 days of 1% DSS oral administration (in the drinking water), caused the loss of about one-third of mucosal crypts. When *Nrf2* KO mice underwent the same treatment, the loss of crypts was significantly greater at about two thirds. This increased vulnerability of *Nrf2*^{-/-} mice was associated with a net lower expression of antioxidant/phase II detoxifying enzymes, such as hemeoxygenase-1 and glutathione-S-transferase Mu-1. But, even more importantly, the expression of genes coding for key pro-inflammatory molecules, such as IL-1, IL-6, TNF α , iNOS and COX-2, was significantly increased in the colonic mucosa of *Nrf2*^{-/-} mice versus the wild-type mice (119).

The *in vitro* and *in vivo* experimental findings of Osburn and colleagues further strengthened the evidence of negative regulation exerted by Nrf2 on the expression of inflammatory molecules and on intensity of inflammatory reactions also in the gut mucosa. The group's main aim was to investigate the contribution of colonic inflammation in inducing colonic pre-neoplastic lesions after a single

initiating dose of azoxymethane, using *Nrf2*^{+/+} and *Nrf2*^{-/-} mice. Two weeks after intraperitoneal injection of azoxymethane, mice were given 1% DSS in the drinking water, for six or seven days. Inflammation was only characterized at the end of DSS treatment, while the possible onset of pre-neoplastic lesions was evaluated three weeks later. DSS-induced colitis was much more severe in *Nrf2*^{-/-} mice than in wild-type mice, with particularly marked damage to the mucosa, which was extensively infiltrated by leukocytes, mainly macrophages and neutrophils (194).

Consistently, at the end of DSS treatment, significantly higher transcription of the pro-inflammatory genes IL-1 β , TNF α and IL-12p40 was detectable in the colonic mucosa of *Nrf2* KO mice than in those with active Nrf2. The morphological evidence of colonic wall infiltration by professional phagocytic cells in *Nrf2*^{-/-} mice was biochemically confirmed when a net increase of MPO was found in the colon of these animals. Other relevant findings were a net rise of 3-nitrotyrosine-positive macrophages and of lipid peroxidation products in the colonic mucosa of *Nrf2*^{-/-} mice versus *Nrf2*^{+/+} mice (194).

Besides confirming and expanding the finding of a more marked inflammatory reaction in the colon from *Nrf2* KO mice versus wild-type mice, the same study reported that Nrf2-deficient mice were also more susceptible to inflammation-associated pre-invasive colorectal carcinogenesis versus wild-type mice. In fact, three weeks after having stopped DSS treatment, the number of aberrant crypt foci in the colon of azoxymethane plus DSS *Nrf2*^{-/-} mice was three times higher than it was in azoxymethane plus DSS treated wild-type mice (194). Subsequently, adopting the same cancer initiation treatment, i.e. intraperitoneal injection of a single dose of azoxymethane, two weeks interval, then one week of DSS, but in this case with a follow-up of a further 17 weeks (20 weeks in total), Khor and colleagues were able to evaluate the incidence of colon tumors in *Nrf2* KO and wild-type mice, and found a marked difference in the tumorigenesis rate: only 53% of wild-type mice versus 92% of KO mice, showed macroscopic tumor. Of note, while in *Nrf2*^{+/+} tumor-bearing mice, 80% were adenomas and 20% were adenocarcinomas, in the *Nrf2*^{-/-} mice, incidence of benign versus malignant colon tumors was the converse, i.e. 20% were adenomas and 80% were adenocarcinomas (119).

With regard to oxidative stress, a biochemical condition that significantly exacerbates the expression of IBD, NOX is considered to be one of the main cellular sources of ROS; using a mouse model of LPS-induced sepsis, Nrf2 has recently been shown to be essential for the physiological regulation of this enzyme's activity. According to the researchers involved, the transcription factor down-regulates the activity of protein kinase C (PKC), which is a redox-sensitive kinase, as well as reducing the consequent activation of NOX, by increasing intracellular levels of antioxidants, in particular of GSH. This would at least partly explain why Nrf2 deficient mice display increased generation, as well as increased steady-state-levels, of ROS (129).

The overall primary function of Nrf2 in moderating and controlling the expression and extent of inflammatory processes has been carefully analysed in a recent review (122). Disruption of Nrf2 activity was shown to allow an exaggerated production of pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF α , to take place; on the contrary, activation of this transcription factor efficiently down-regulated the expression of key chemokines, like MCP-1 and IL-8, adhesion molecules, like VCAM-1, metalloprotease-9, as well as down-regulating COX-2-dependent inflammatory reactions (122).

On the basis of the available literature, it might be concluded that NF-kB up-regulation initiates and sustains a variety of pro-inflammatory reactions, whereas Nrf2 orchestrates a number of antioxidant-related biochemical events; together these might provide a fundamental quenching and negative regulation mechanism of inflammation itself.

VII. THERAPEUTIC CONSIDERATIONS

It is now widely accepted that intestinal damage in IBD is caused by an altered immune response to the luminal environment. This alteration results in a loss of tolerance and therefore the establishment of a chronic inflammatory response. Based on the latest findings, the treatment of IBD patients focuses on counteracting this exaggerated inflammatory response.

The primary aim of IBD treatment is to induce and maintain remission and to prevent relapse. The classical therapeutic approach entails conventional *anti-inflammatory agents*, such as 5-aminosalicylates and corticosteroids, which usually provide significant suppression of inflammation

and rapid relief of symptoms. However, IBD management requires long-term treatments that often bring side effects, or may even lead to drug refractoriness or intolerance. Furthermore, these anti-inflammatory drugs do not prevent intestinal fibrosis, stricture formation, and consequent need for surgical intervention, especially in CD (44). These patients often respond inadequately to commonly-used steroids, a condition which leads to a poor quality of life.

Immune modulators, namely thiopurines, cyclosporin A or methotrexate, are currently widely used in treating IBD, in an attempt to induce immunosuppression. However, these drugs do have cytotoxic effects and increase the risk of adverse hematological, hepatic, and gastrointestinal events, as well as the susceptibility to highly infectious diseases. Although immunosuppressive therapy is commonly used, together with anti-inflammatory drugs, it requires close monitoring and personalized application (41, 117).

Because of the growing incidence of IBD world-wide, a more targeted therapeutic approach is increasingly demanded by the medical profession. Recent pathophysiological studies have led to more specific drugs being used, including many biological drugs, which have revolutionized the treatment of IBD, showing clinical benefits in maintenance therapy.

Biological therapy is focused on the inflammatory response, and aims to antagonize pro-inflammatory molecules. Inflammatory cytokines are thus the most logical targets in IBD treatment, and many studies have been designed to synthesize specific cytokine inhibitors. In this connection, the two cytokines TNF α and IL-1 β have been widely studied. Currently, the use of TNF α blockers is the only licensed biological therapy for IBD, in particular for CD. Figure 10 shows the stages of today's therapeutic approaches to IBD management.

However, new targeted therapeutic approaches are now being suggested by the rapidly improving knowledge of the pathogenesis of IBD, as it becomes clear that a primary contribution to the disease progression is made by exaggerated and dysregulated NF- κ B signaling and overproduction of ROS. Indeed, studies on both *in vivo* and *in vitro* models of intestinal inflammation have strongly implicated oxidative reactions in the mucosal barrier injury. Modulating NF- κ B signaling and targeting ROS overproduction sites, in particular plasma-membrane NADPH oxidase and

mitochondria, may soon provide important new therapeutic options to lessen intestinal damage. Lastly, recent clinical trials have outlined the potential efficacy of chronic dietary supplementation of polyphenols, which markedly down-regulate the expression of inflammatory cytokines and adhesion molecules, as well as that of nutritional diets including the antioxidant vitamins, which prevent the reactivation of IBD. Thus a diet rich in certain polyphenols and in the vitamins A, C, and E, may be recommended to IBD patients, not only during active disease, but particularly in the remission state.

VII A. TNF α -mediated NF- κ B signaling

TNF α plays a key role in the induction of intestinal inflammation, through regulating NF- κ B. The use of anti-TNF α antibodies in the IBD therapy has recently generated considerable interest. Several TNF α blockers are currently licensed for IBD treatment, namely infliximab, adalimumab, and certolizumab, which have been approved by the US Food and Drug Administration for the treatment of moderate-to-severe CD, with inadequate response to standard medications (23). Infliximab is a chimeric monoclonal IgG1 antibody, which binds with high affinity both the soluble and the transmembrane forms of human TNF α in the intestinal mucosa, thereby neutralizing its effect. Adalimumab is a complete human immunoglobulin G1, which specifically blocks interactions with TNF α receptors. Certolizumab is a monoclonal antibody, with the Fab fragment of human anti-TNF α combined with polyethylene glycol.

Infliximab appears to be the most effective of these various TNF α blockers, especially in fistulizing active CD. A Crohn's disease Clinical study Evaluating infliximab in a New long-term Treatment regimen (ACCENT I) performed from 1999 to 2000, on 573 CD patients from 55 sites in North America, Europe, and Israel, provided the first significant evidence of the efficacy and safety of repeated infusions of infliximab, in patients who responded after an initial infusion of this compound (86). A second ACCENT II study demonstrated the efficacy of infliximab in sustaining fistula closure, in patients with active fistulizing CD (who had single or multiple draining fistulae) with a higher likelihood of abscess development (220). More recently, a randomized, double-blind Study of Biologic and Immunomodulator Naïve Patients in Crohn's Disease (SONIC) trial, conducted in 92

centers from March 2005 to November 2008, in 508 adult patients with moderate-to-severe Crohn's disease (who were naïve to both agents) treated with infliximab and/or azathioprine, showed that the combination therapy was more effective at inducing remission and mucosal healing (45).

However, infliximab therapy brings the risk of significant side effects or refractory disease, especially in patients treated with infliximab who also receive concomitant immunomodulators, such as azathioprine or mercaptopurine, as well as corticosteroid therapy (57, 135). Combined therapy has been associated with significant toxicity, especially with an increased risk of serious infections. Thus, patients with latent infections, such as tuberculosis or hepatitis B, should be excluded or treated before starting biological therapy (268). In addition, long-term exposure to infliximab has been *per se* associated with a loss of response, due to a dysregulation of T cell subsets' activation (239). Treatment with infliximab for a long period can result in the development of antibodies by the host against this molecule, reducing the duration of response to treatment, and increasing the risk of adverse events (54). A multicenter study on corticosteroid-refractory UC patients, in whom cyclosporin had failed, showed that the use of infliximab avoids colectomy in a relatively high percentage of patients, although 23% developed adverse events (38).

Recently, numerous studies using anti-TNF α agent adalimumab as therapy have provided evidence of clinical improvement in CD patients in whom previous infliximab therapy had failed (47). Adalimumab therapy showed a sustained steroid-free remission and fistula healing in Canadian patients with moderate-to-severe Crohn's disease (199).

TNF α signals initiate through binding to its surface receptors, TNF-R1 (ubiquitously expressed), and TNF-R2 (mainly found in immune cells). Many cell types co-express the two TNF receptors. TNF α induces their trimerization and results in the activation of apoptosis or inflammation. The induction of different processes depends on the recruitment of signal transducers, which activate three distinct down-stream effectors: Fas-associated death domain (FADD) protein, which is responsible for apoptosis recruiting and activating caspase 8, RIP1, which appears to be a key effector in the TNF-R1 dependent NF- κ B activation, and the TNF-receptor-associated factor 2 (TRAF2),

which is activated by both TNF-R1 and TNF-R2, and leads to activation of their common cell signals IKK, JNK and p38 MAPK. (18).

The key factor in regulating the balance between apoptosis and inflammation is the redox transcription factor NF- κ B. Upon TNF α stimulation, the different components of TNF α signaling undergo post-translational modifications, namely ubiquitination and phosphorylation, which activate and amplify the inflammatory response mediated by NF- κ B, which in turn suppresses the apoptotic cascade. Conversely, the duration of NF- κ B activation is negatively controlled by the induction of different inhibitors associated to the TNF α signal. Among the NF- κ B negative regulators identified, a TNF-R2a site located in the carboxyl terminus of TNF-R2 has been found to block TNF α signaling, binding TRAF2 (300). Furthermore, ubiquitin ligase A20, which is also called TNF α -induced protein 3, is said to play an important role in the down-regulation of TNF α -NF- κ B axis. A20 appears to block the recruitment of RIP and TNF-R1-associated death domain (TRADD) to TNF-R1 complex; RIP itself may be degraded by A20 (150). Interestingly, A20 has recently been found to be up-regulated by TLR signaling during inflammation, attenuating TLR response in a negative feedback loop (195). These findings suggest the potential clinical importance of A20, as a new target molecule playing a crucial role in the management of intestinal inflammation. The treatment of IBD patients with TNF α antibodies is indeed capable of inhibiting NF- κ B (193).

Recently, several attempts to reduce NF- κ B and inflammatory cytokines have been made using anti-oxidant compounds such as phenolic acids. For instance, sylimarin was found to reduce TNF α and NF- κ B activation in experimental colitis (169), and black tea restored adhesion molecule activation, by blocking NF- κ B and the JNK cell pathway in IEC treated with LPS (238). Mitochondrial prohibitin (PHB) (for details see below) has been shown to decrease TNF α and NF- κ B, both in IEC and in colonic mucosa of prohibitin transgenic mice (with a two fold increase in PHB expression in colon mucosa), suggesting this molecule may be protective against intestinal injury in IBD (262).

It has been reported that TNF α potently activates NOX1, inducing transactivation of the *NOXO1* gene in human colon epithelial cells; hence, this oxidase is a potential target in treating colon

inflammation (140). Furthermore, TNF α was also found to influence NF- κ B, by enhancing ROS production mediated by another NADPH oxidase isoform, NOX2 (151).

Future perspectives in IBD therapy involving TNF α signaling should focus on the various molecules that lead to induction of NF- κ B activity, rather than using antibodies directed against TNF α itself.

VII B. The IL-1 family and inflammasomes as ROS-dependent targets

Different members of the IL-1 family, such as IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1Ra), IL-18 and IL-33, are known to be actively involved in innate and adaptive immune responses. In particular, IL-1 β and IL-18 appear to be of great interest in the pathogenesis of intestinal inflammation. IL-1 β has been widely investigated for its strong pro-inflammatory effects, which in many cases overlap those of TNF α , in terms of cell type and signaling molecules involved, despite the fact that there is no homology with any group of proteins activated by TNF α . IL-1 β has an anti-microbial function and regulates systemic reactions, i.e. fever and septic shock, as well as local inflammatory response. IL-18, like IL-1 β , must be processed by caspase-1 in order to be activated.

The concept that intestinal inflammation induced by IL-1 β and IL-18 is only a contributory factor in tissue damage is now being revisited, based on recent reports on DSS- and TNBS-induced colitis-associated tumorigenesis, which suggest that the NLRP3 inflammasome and IL-1-related signaling are critically involved in protection against colorectal tumor development (4, 296). However, an altered response to luminal agents, with excessive inflammatory reactions, could maintain the inflammasome response harmfully active, which in turn might influence the activity of IL-1 β and IL-18. High levels of these interleukins have, indeed, been detected in the inflamed mucosa of numerous IBD patients. Increased expressions of IL-18 and of its binding protein have recently been observed in the chronically-inflamed mucosa of children with CD, and in the serum of adults with active CD, but not in UC patients (143, 177). The levels of IL-1 β have been found to be increased in macrophages of DSS colitic mice, in which ROS generation was necessary for p38 MAPK and ERK1/2 activation, and consequently for IL-1 β production. Furthermore, IL-1 β release

and caspase-1 activity induced by DSS treatment were significantly inhibited by MAPK kinase inhibitors, and by NAC, a precursor of the antioxidant GSH (141). Extra-intestinal complications, such as thrombosis, have been related to IL-1 β enhancement in mice with DSS-induced colitis (293). As discussed at length above, the implication of IL-1 β in the pathogenesis of IBD gained new importance with the discovery of the NLRP3 inflammasome.

ROS are crucial for the secretion of IL-1 β via inflammasome activation. The experimental model of DSS-induced colitis in mice, both wild type and deficient in different components of NLRP3, showed that ROS signaling is indispensable to activate the inflammasome-dependent IL-1 β production by macrophages (19). Mitochondria are important cellular sites actively involved in ROS generation, which leads to NLRP3 inflammasome activation (240). It has been proposed that exposure to stressors may activate NLRP3-IL-1 β , either through NF- κ B induction or through the interaction between ROS and a Trxip. ROS can be generated by direct exposure to PAMPs or DAMPs, but also in response to ATP-dependent potassium efflux and phagosomal or lysosomal damage. Trxip may directly interact with the redox domain of antioxidant Trx, and might function as a negative regulator of Trx reductase. In the presence of oxidative stress, Trxip dissociates from Trx and binds to NLRP3, which then undergoes conformational changes and activation (298). In UC, the mucosal production of IL-1 β , H₂O₂, and NO may contribute to the impaired Ca²⁺ release, decreasing sigmoid smooth-muscle contractility (36).

The intestinal epithelium thus appears to be highly sensitive to both decreased and increased expression of the inflammasome effectors, IL-1 β and IL-18, which can culminate in severe mucosal injury. In this connection, synthetic antibodies against IL-1 β and IL-18 have been developed, in an attempt to reduce the severity of colitis in animal models (118, 232, 293).

The IL-1Ra is considered to be an important cytokine, whose action comprises competitive inhibition of the binding of IL-1 β to its receptor. A low ratio between IL-1Ra and IL-1 β has been found in explant cultures of colonic biopsies from both UC and CD (60). Different polymorphisms in gene sequences of IL-1 β and IL-1Ra have been identified in IBD populations (48, 291). IL-1Ra has been shown to have beneficial effects in experimental models of colitis (77, 214), and has been

considered for use in managing intestinal inflammation. At present, sIL-1R, a non-glycosylated recombinant analogue of secreted IL-1Ra known as Anakinra, has been used in human rheumatoid arthritis and other rheumatic disorders, but solid evidence regarding its application in the therapy of IBD is yet to be obtained (16).

VII C. NADPH oxidase as target of the pathogen recognition pathway

The recent findings concerning the involvement of different NADPH oxidase isoenzymes, in inducing ROS in NLRP3, suggest that NOXs may be possible targets of IBD therapy (162, 174).

Efficient bacterial recognition, processing and killing require a discrete production of bactericidal ROS, via NADPH oxidase enzyme complexes. These are complex enzymes consisting of cytosolic and membrane subunits, which assemble variously to form tissue-specific isoforms of NOX and Dual Oxidase (DUOX). In particular, NOX1 and DUOX2 are present in epithelial cells, whereas NOX2 is mainly found in phagocytes. Notably, NOX and DUOX have been shown to regulate the number of bacteria in response to commensal and infectious microbes in the intestine of *Drosophila melanogaster* (112). These enzymes are closely involved in the autophagy mechanisms activated by ROS generation. NOD2 signaling involves ROS generation via DUOX2 and the activation of downstream inflammasomes and NF- κ B: colon tissue levels of ROS in wild-type mice stimulated with muramyl dipeptide were increased, while in muramyl dipeptide-stimulated *NOD2*^{-/-} mice, ROS generation was abrogated (154).

Hyper-activation of different NOX/DUOX isoforms, highly expressed in mucosal cells, may inflict damage to the host tissue. The association between susceptibility to develop gastrointestinal diseases and altered function of phagocytic and non-phagocytic NADPH oxidase isoforms, has recently been hypothesized (40, 163, 216). Lymphocytes present in the mucosal lesions of CD and UC were also found to be strongly positive for NOX1 (248). A significant increase of DUOX2 mRNA expression was found in colonic biopsies from both CD and UC patients (154).

NOX family stands as a promising target for new IBD therapeutic strategies, despite the fact that presently available NOX inhibitors are not fully specific. In relation to this, a recent study has

identified a potent, highly selective inhibitor of NOX1, namely the compound ML090 (CID-616479) (31).

VII D. The mitochondrial target prohibitin

Mitochondria are another primary site of ROS generation in the immune response against dietary antigens. The recent characterization of a mitochondrial antiviral signaling protein, which mediates NF- κ B and IRF3 activation, supports the evidence of active involvement of the mitochondria in microbial defense (229). TLR1, TLR2 and TLR4 can also increase ROS production in phagosome mitochondria (286). Intact mitochondria with intense respiratory activity are necessary for NLRP3 induction, in order to control bacterial recognition and immune response (115, 240). Furthermore, mitochondrial DNA may play an active role by contributing to the secretion of IL-1 β and ATP in NLRP3 activation (182, 240).

Mitochondrial derangement may be involved in intestinal inflammatory diseases. Interestingly, a complex regulating network has been hypothesized between mitochondrial machinery and NLRP3: NLRP3 is suggested to efficiently sense high concentrations of ROS produced by malfunctioning mitochondria, thus causing excessive activation of the inflammatory response. This points to an important role of mitochondrial derangement in the development of IBD (299). Marked mitochondrial damage in IEC and mucosal injury in terms of increased protein oxidation, have been found in IBD patients (64). In addition to that, increased TNF α expression, with transient enhancement of ROS production leading to mitochondrial derangement, has been observed throughout all the intestinal layers of neonates with necrotizing enterocolitis, and in rat IEC treated with TNF α ; the possible role of TNF α -induced ROS in the activation of mitochondrial autophagy was stressed (15). An increase in mitochondrial respiratory chain complex IV, with an enhancement of lipid peroxidation-end products, was shown in the colon of DSS-treated rats (51).

Recently, functional alterations of PHB, which are evolutionarily-conserved and ubiquitously-expressed membrane proteins mainly localized in the mitochondria, have been suggested in various pathological processes characterized by oxidative modifications (187). PHB levels are decreased in

IEC during oxidative stress, and in the IBD inflamed mucosa (97, 260). Low levels of the PHB complex, with changes in PHB mitochondrial expression and localization, resulted in increased ROS generation and sensitivity to free radicals (242, 264).

The eukaryotic mitochondrial PHB complex comprises two subunits, PHB1 and PHB2, which assemble into a ring-like complex, and anchor to the inner mitochondrial membrane through N-terminal hydrophobic regions present in both PHB1 and PHB2. PHB1 appears to be present in larger amounts than PHB2 in mitochondria, and is mainly involved in maintaining mitochondrial morphology and distribution. PHB complex contributes to the stabilization of the mitochondrial genome (281); it is implicated in regulating membrane protein degradation by the mitochondrial *m*-AAA protease (244), and can function as a holdase/unfoldase chaperone that holds and stabilizes unassembled membrane proteins (186). PHB complex plays a role in mitochondrial morphogenesis (167), functioning as a scaffold that recruits membrane proteins to a specific lipid environment; this function ensures the integrity and functionality of the mitochondrial inner membrane (11, 196).

PHBs are also present in other cellular compartments, including nucleus and plasma-membrane, the specific location depending on cell type and situation. PHB complex has been identified as a component of the cell-surface-associated molecular complex in human intestinal Caco-2 cells, binding to Vi. The interaction of Vi with this complex makes *Salmonella typhi* resistant to phagocytosis and to the action of serum complement. PHB complex thus inhibits the inflammatory response to *Salmonella typhi* in the intestine. In addition, this interaction on Caco-2 cells causes a reduction in IL-8 secretion by involving the MAPK pathway (230). This finding suggests that cell-surface-bound PHB complex might be an important target for the modulation of the inflammatory response by various molecules. In this connection, it may be hypothesized that small molecules, peptides or monoclonal antibodies, which interact with the PHB complex on intestinal cells, might play a therapeutic role in a variety of gastrointestinal diseases, including IBD, by reducing or inhibiting the inflammatory responses (170).

Since IBD is associated with increased oxidative stress and mitochondrial dysfunction, PHB1 could play a role in combating oxidative stress in the gut, in particular by inhibiting GSH depletion.

Indeed, IEC overexpressing PHB have been found to show an increase in GSH levels and an induction of glutathione-S-transferase π expression (260).

Furthermore, TNF α (96) may contribute to decreasing PHB1 mucosal levels: TNF α has been found to reduce PHB1 levels in cultured IEC and in animals (262). TNF α activates the transcription factor NF- κ B, which decreases PHB expression. Overexpression of PHB1 in these cells protects against the deleterious effects of TNF α and NF- κ B on the intestinal barrier function by inhibiting TNF α -induced NF- κ B (262). This finding suggests that PHB1 may be crucial in modulating intestinal inflammatory processes.

Contrary to TNF α , IL-6 has been shown to up-regulate PHB expression in IEC and in the intestine; cytokine increases PHB mRNA and protein and induces PHB promoter activation. The IL-6 response element site in the PHB promoter is required for maximal basal promoter activity and responsiveness to IL-6 (261). Thus, IL-6 may protect intestinal mucosa from oxidative stress and inflammation by increasing PHB levels and inducing antioxidant defenses. In support to this observation, *IL-6*^{-/-} mice show reduced PHB expression in the colon compared to wild-type mice (261), and suffer more severe erosion of the intestinal epithelium during experimental colitis (257).

PHB1 levels in the IEC of transgenic mice overexpressing PHB1 can be restored during inflammation (263); in these mice, it has been observed that prohibitin acts as an antioxidant, by decreasing oxidative stress and regulating the antioxidant response. Indeed, PHB has been shown to sustain activation of the transcriptional regulator of antioxidant responses Nrf2, and to protect from inflammation-associated oxidative stress (263).

To demonstrate the therapeutic efficacy of PHB restoration for human IBD, adenovirus infection and nanoparticle delivery of biologically-active PHB1 have been tested in mice during experimental colitis: both methods of delivery have resulted in increased PHB1 levels in colon epithelial cells and in reduced severity of induced colitis (264).

Taken together, all these *in vivo* and *in vitro* experimental studies consistently indicate that the PHB complex tends to protect IEC from inflammation-associated oxidative stress, thereby counteracting the mucosal barrier disruption induced by increasing steady-state levels of oxidants. On

these bases, the restoration of physiological levels of PHB1 in the intestinal mucosa affected by IBD appears to be a very promising and interesting approach (259).

VII E. Suitable dietary regimens in IBD patients.

Over recent years, a small number of clinical trials have been carried out to evaluate the efficacy of special nutritional regimens in preventing relapse in patients with quiescent IBD. The only study thus far performed in a randomized, multicenter, placebo-controlled manner, which employed polyphenols to prevent IBD reactivation, showed that chronic dietary administration of curcumin plus sulfasalazine for six months, to patients (n=45) with ulcerative colitis, significantly reduced the percent incidence of disease relapse (5%) versus a similar group of patients (n=44) receiving only the anti-inflammatory drug (20%). The two groups of UC patients were then followed for six further months, during which only the anti-inflammatory therapy was administered, without curcumin. At the end of this second period, the number of relapses was quite similar in the two groups (84).

More recently, other clinical trials have used polyphenols as a dietary supplement to counteract or prevent overexpression of inflammatory cytokines and adhesion molecules, not in IBD patients but in individuals with a high risk of developing atherosclerosis. In 67 male high-risk volunteers, who drank a standardized amount either of ethanol or of dealcoholized red wine, the phenolic extract from red wine was shown to down-regulate expression of certain adhesion molecules and cytokines in blood mononuclear cells, in particular intercellular adhesion molecule (ICAM)-1, E selectin and IL-6 (39).

Another type of so-called “nutritional therapy” employed a comprehensive mixture of aminoacids and vitamins, including the antioxidant vitamins A, C, and E (elemental diet patented as Elental), for home treatment of quiescent IBD, with the aim of delaying disease reactivation. Fifty CD patients in remission were randomly divided into two subgroups, one receiving a free diet and the other receiving a diet supplemented with the aminoacids/vitamins. After two years’ follow-up, the number of cases of disease reactivation was reduced to half in patients receiving nutritional therapy (34% relapses) versus those on a free diet (64% relapses) (250). Use of the same elemental diet has

recently been compared to the frequently-used drug 6-mercaptopurine, as maintenance therapy of CD in remission. After two years' follow-up, the number of patients (n=32) receiving the elemental diet, rich in vitamins and aminoacids, showed a relapse rate (51%) quite similar to that of CD patients (n=30) receiving 6-mercaptopurine instead (40%). The researchers concluded that suitable nutritional therapy could achieve comparable efficacy to purine antimetabolites in preventing reactivation of CD (85).

VII F. Lipoxins and resolvins: new drug candidates for IBD treatment

There is considerable interest in the relation between fatty acids and gut inflammation. During acute inflammation, resident cells in the injured tissue produce soluble pro-inflammatory mediators, such as eicosanoids deriving from ω 6-AA. This regulates early events in the inflammatory response, promoting the influx of neutrophils from the blood and their phagocytic activity. The resolution of acute inflammation is an active programmed process with a self-limited response of leukocytes, which provides a temporal switch in lipid mediator production within the family of eicosanoids, from pro- to anti-inflammatory PGs and LTs, and also their fatty acid precursors. Indeed, during the latter phase of inflammation, changes occur in the quantity and quality of the leukocyte infiltrate. In this phase, neutrophil numbers decrease, dying by apoptosis, while lymphocytes and monocytes/macrophages predominate, and combine to resolve inflammation by means of the endogenous generation of specific ω 3- and ω 6-PUFA derivatives with anti-inflammatory and pro-resolving activity, including lipoxins (LXs) and resolvins (Rvs) (227).

Chronic inflammation is now believed to be the evolution of unresolved acute inflammation that can no longer be controlled. To prevent chronicization, it appears to be more important to improve the resolution of the process, rather than inhibiting inflammation by using anti-inflammatory glucocorticoids (226).

Endogenous LXs and Rvs have aroused interest, because of their ability to promote the clearance of apoptotic cells by macrophages, and to limit the infiltration of pro-inflammatory leukocytes, which eventually leave the inflamed site. LXs and Rvs are also generated after acetyl

salicylic acid treatment, providing a basis for understanding a novel mechanism of action of both nonsteroidal anti-inflammatory drugs (NSAIDs) and dietary ω 3-PUFA supplementation.

Their function in mediating the resolution of inflammation makes them new promising candidates for regulating inflammation-associated disorders like IBD.

LXs are generated through transcellular biosynthesis, in which cell-cell interactions activate 15-LOX in different cell types at the inflammation site, to produce 15(S)-hydroperoxyeicosatetraenoic acid from AA. This compound is rapidly incorporated in leukocytes, and converted by 5-LOX and hydrolase into LXA4 or LXB4.

Endogenous LX analogs are also produced after exogenous administration of aspirin (but not with other NSAIDs), which irreversibly acetylates COX-2 in endothelial cells or leukocytes, switching AA derivatives from pro-inflammatory PGs and LTs to LXs. Acetylated COX-2 is still active, but generates 15(R)-hydroxyeicosatetraenoic acid that, in turn, in endothelial or epithelial cells is converted by 15-LOX into the potent anti-inflammatory 15-LXA4 epimer, known as 15-*epi*-LXA4 or aspirin-triggered lipoxin (ATL) (226, 243).

LXs limit the number of neutrophils in inflamed areas: they stimulate macrophages to ingest and clear neutrophilic apoptotic bodies, and promote only the infiltration of non-inflammatory monocytes, which produce anti-inflammatory and pro-fibrogenic cytokines. In particular, the widely studied LXA4 signals, through a G protein-coupled receptor, to inhibit chemotaxis and transmigration, and down-regulates pro-inflammatory pathways, such as ERK and p38 MAPK; it limits the activity of AP-1 and NF- κ B transcription factors, NOX-dependent ROS production, and inhibits the expression of inflammatory cytokines such as IL-8 and TNF α (9, 108, 137). Synthetic metabolically-stable LX analogs have been made to mimic anti-inflammatory LX actions, and prolong its active effect. A stable LXA4 analog has been found to down-regulate IL-8 mRNA expression via NF- κ B suppression T84 polarized IEC infected by *S. typhimurium*, which can be considered an *in vitro* model of inflammation (81). The therapeutic anti-inflammatory effect of LXA4 analogs has been verified on the DSS-induced murine colitis model, in which oral administration of this active analog led to the attenuation of colitis and down-regulation of pro-inflammatory gene expression (81).

Similar effects are reported for a synthetic analog of ATL examined in TNBS-induced experimental colitis. Oral administration of this analog decreased mucosal mRNA level of COX-2, macrophage inflammatory protein-2 (MIP-2), TNF α , IL-2 and IFN γ , showing anti-inflammatory efficacy similar to that observed with prednisolone (71).

Studies in animal models of colitis, in which exogenous LX analogs resolve inflammation, support the hypothesis that LX deficiency plays a role in IBD. Mangino and colleagues found a decrease of LX production in colonic mucosal tissue from UC patients, compared to both organ donors and patients affected by slow-transit-time constipation. They found a decrease of epithelial 15-LOX in UC biopsies, suggesting defective biosynthesis of LXs in these patients (160).

These findings were strengthened by a recent study on humans that found elevated LXA4 biosynthesis in mucosal biopsies from UC patients in medically-induced remission, confirming the hypothesis that LXA4 exerts protective and/or reparative action in human intestinal inflammation (276).

Resolvins are potent bioactive compounds, which exert their anti-inflammatory function by blocking trans-endothelial leukocyte migration and infiltration. They derive from the degradation of eicosapentanoic (EPA) and docosahexaenoic (DHA) ω 3-PUFAs through COX-2 and LOX enzymes. RvE-series has been identified in endothelial cells expressing COX-2 treated with aspirin: as in the case of LXs, Rvs are generated by acetylated COX-2, which converts EPA to 18(R)-hydroxyeicosapentanoic acid, and eventually to RvE1 or RvE2, by leukocyte 5-LOX. RvD-series is generated from DHA by 15-LOX or aspirin-derived acetylated COX-2, and 5-LOX, through intermediates 17(S)-hydroxydocosahexaenoic acid or 17(R)-hydroxydocosahexaenoic acid (in the presence of aspirin). Notably, biosynthesis of RvE1 can be also initiated by P450-like enzymes in bacteria (228).

Resolvins may provide the molecular means underlying ω 3-PUFA's beneficial effects in IBD. Exogenous RvE1 administration protects against the development of TNBS-colitis in mice. 100 ng of RvE1 reduced leukocyte peritoneal infiltration by 50%, and increased survival and histological scores. Notably, a significant reduction in TNF α , IL-12-p40 subunit, iNOS, and COX-2 was observed in mice

that had received RvE1 (10). It has recently been reported that RvE1 treatment in mice with DSS-induced colitis led to less severe histological features of colitis, with a reduction in body-weight loss. In this experimental model, RvE1 administration reduced NF- κ B phosphorylation and expression of the NF- κ B-dependent pro-inflammatory mediators TNF α , IL-1 β , and IL-6. (105). Anti-inflammatory effects of the aspirin-triggered RvD1, of its precursor 17-hydroxy-DHA, and of RvD2 were demonstrated in DSS- or TNBS-induced colitis, reducing colonic cytokine levels as well as mRNA expression of NF- κ B and adhesion molecules (22).

Elucidating the molecular basis of ω 3-PUFA action in generating local mediator Rvs, and their effectiveness in clinical applications as anti-inflammatory therapy in IBD, are still major issues; data currently available only concern experimental models. Transgenic *fat-1* mice with high tissue levels of ω 3-PUFA, because they have been engineered to express *C. elegans fat-1* gene encoding for ω 3 fatty acid desaturase, showed a significant production of Rvs. Further, the same experimental model was protected against DSS-induced colitis, leading to a manifest reduction in tissue injury inflammation, with significantly low amounts of TNF α , IL-1 β cytokines, as well as reduced NF- κ B activity (99).

A diet involving the intake of the recommended ω 6/ ω 3-PUFA ratio could undoubtedly help to delay or prevent the progression of IBD. However, clinical and epidemiological studies on the role of PUFA supplementation in dampening inflammation in IBD, through the local production of anti-inflammatory lipid derivatives, still do not afford conclusive results (35, 287).

VIII. CONCLUSIONS

This review has examined the current understanding of the mechanisms regulating intestinal barrier integrity, as well as its pathologic alterations during IBD development, with special emphasis on the pathogenetic role played by the multiple redox changes occurring during development of these diseases. The main indications and suggestions for targeted therapy of IBD arising from recent molecular studies have been examined and discussed.

The experimental studies carried out in recent years on the pathogenesis of IBD, together with the observational studies carried out so far on human patients, have defined the main cellular and molecular events underlying the onset and development of this frequent disease, whose incidence is increasing world-wide.

In IBD, the host's impaired ability to properly recognize and process the molecular signals activated by luminal antigens results in a chronic disease process, in which phagocytes and lymphoid cells are the key players. Together, these cells overproduce cytokines, inflammatory eicosanoids, and reactive oxidant species that, in turn, decrease cell and tissue antioxidant defenses, provoking a net imbalance of the local redox state toward oxidation. In addition, unbalanced ROS and RNS production exacerbates the inflammatory process, because of the marked pro-inflammatory properties of these products and their derivatives, in particular the highly-diffusible lipid oxidation products. A vicious circle then occurs between inflammation and oxidant species, amplifying the overall pathological process and hampering its resolution.

With regard to the markedly altered cell signaling that characterizes molecular and cellular interactions in the affected intestine, a pivotal role is now known to be played by redox-sensitive transcription factor NF- κ B, because of the many inflammatory cytokines this factor stimulates at the transcriptional level.

Interestingly, the modern approach to IBD pathogenesis, together with the recent progress in molecular medicine, point to treatment of the oxidative stress associated with IBD not by means of simple (and often ineffective) supplementation with antioxidants, but rather by designing a proper dietary intake of specific lipids with molecular modulation of the main sources of reactive species, i.e. NOXs, NOSs, mitochondria and inflammasomes.

Treatment of IBD with biological drugs is a new and promising therapeutic frontier, notwithstanding the fact that only TNF α blockers have yet been approved for the management of patients with moderate/severe CD, who are unresponsive to conventional therapies. Correct modulation of NF- κ B-driven gene expression, suitable prohibitin-controlled generation of mitochondrial ROS, plus a similarly adequate quenching of NOX-dependent ROS generation and

membrane lipid oxidation, should result in better, and perhaps more personalized, management of this very common disease process. A therapeutic approach that is unlikely to eradicate the disease *per se* but will, in the not-too-distant future, undoubtedly limit its progression and expansion (Figure 11).

Acknowledgments

The authors thank the Italian Ministry of University, PRIN 2008, PRIN 2009, the Piedmontese Regional Government (Ricerca Sanitaria Finalizzata 2008, 2008 II, 2009), the CRT Foundation, Turin, and the University of Turin, Italy, for supporting this work.

Disclosure statement: The authors state that no competing financial interests exist in the present submitted manuscript.

List of abbreviations

7 α -OH: 7 α -hydroxycholesterol

7 β -OH: 7 β -hydroxycholesterol

7K: 7-ketocholesterol

8-OHdG: 8-hydroxy-2'-deoxyguanosine

AA: arachidonic acid

AJ: adherens junction

AJC: apical junctional complex

AP-1: activator protein-1

ATG: autophagy-related protein

ATL: aspirin-triggered lipoxin

Bad: Bcl-xL/Bcl-2 associated death promoter

Bcl-2: B-cell lymphoma 2 anti-apoptotic protein

Bim: Bcl-2 interacting mediator of cell death

CARD: caspase activation and recruitment domain

CAT: catalase

CD: Crohn's disease

COX: cyclooxygenase

CpG: cytosine nucleotide next to guanine separated by a phosphate

DAMP: danger associated molecular patterns

DC: dendritic cells

DHA: docosahexaenoic acid

DSS: dextran sodium sulfate

DUOX: Dual Oxidase

EC: endothelial cells

EGF: epidermal growth factor

eNOS: endothelial nitric oxide synthase

EPA: eicosapentanoic acid

ERK: extracellular signal regulated kinase

FADD: Fas-associated death domain protein

FGF: fibroblast growth factor

FLIP: fllice-like inhibitory protein

FOXP3: forkhead box protein 3

GC: goblet cell

GM-CSF: granulocyte-macrophage colony-stimulating factor

Gpx: glutathione peroxidase

GSH: reduced glutathione

GSSG: glutathione disulfide

HBD: human β -defensin

HD: human α -defensin

HLA: human leukocyte antigen

HNE: 4-hydroxynonenal

I κ B: inhibitor of κ B

IAP: inhibitor of apoptosis protein

IBD: Inflammatory bowel disease

ICAM: intercellular adhesion molecule

IEC: intestinal epithelial cell

IFN: interferon

IKK: I κ B kinase

IL: interleukin

iL: intraepithelial lymphocyte

iNOS: inducible nitric oxide synthase

IPAF: IL-1 β converting enzyme-protease activating factor

IRF: interferon regulatory factor

IRGM: immunity-related guanosine triphosphatase M

JAK: just another kinase

JAM: junctional adhesion molecule

JNK: c-Jun N-terminal kinase

L \cdot : lipid radical

LO \cdot : alkoxy radical

LOO \cdot : peroxy radical

LOX: lipoxygenase

LPS: lipopolysaccharide

LRRK2: leucine-rich repeat kinase 2

LRRs: leucine-rich repeats

LT: leukotriene

LX: lipoxin

M: macrophage

MAMPs: microbe-associated molecular patterns

MAPK: mitogen activated protein kinase

MC: microfold cells

MCP-1: monocyte chemotactic protein-1

MDA5: melanoma differentiation-associated gene 5

MIP: macrophage inflammatory protein

MLCK: myosin light-chain kinase

MMP: matrix metalloproteinase

MPO: myeloperoxidase

MUC: mucin

MYD88: myeloid differentiation primary-response gene 88

NAC: N-acetyl-cysteine

NEMO: NF- κ B essential modulator

NF- κ B: nuclear factor- κ B

NLR: NOD-like receptor

NLRP: nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain

NO: nitric oxide

NOD: nucleotide-binding oligomerization domain

NOX: NADPH oxidase

Nrf2: nuclear factor-erythroid 2-related factor 2

NSAID: nonsteroidal anti-inflammatory drugs

Ox-PLs: oxidized phospholipids

PAMPs: pathogen-associated molecular patterns

PC: Paneth cell

PDGF: platelet-derived growth factor

PG: prostaglandin

PHB: prohibitin

PI3K: phosphatidylinositol 3 kinase

PKC: protein kinase C

Plase A₂: phospholipase A₂

PMN: polymorphonuclear cells

PRRs: pathogen recognition receptors

PUFA: polyunsaturated fatty acid

PUMA: p53 upregulated modulator of apoptosis

PYK2: proline-rich tyrosine kinase 2

RANTES: regulated upon activation, normal T cell expressed and secreted

RELM β : resistin-like molecules beta

RICK: RIP-like interacting caspase-like apoptosis regulatory protein kinase

RIG-I: retinoic acid inducible gene I

RIP: receptor-interacting protein

RLRs: RIG-I-like receptors

RNS: reactive nitrogen species

ROS: reactive oxygen species

Rv: resolvin

SOD: superoxide dismutase

STAT: signal transducer and activator of transcription

TFF: trefoil factor

TGF β : transforming growth factor beta

Th: T helper

TIR: Toll-IL-1-resistance

TJ: tight junction

TLR: toll-like receptor

TNBS: trinitrobenzene sulfonic acid

TNF-R: tumor necrosis factor receptor

TNF α : tumor necrosis factor α

TRADD: tumor necrosis factor receptor type 1-associated death domain

TRAF2: TNF-receptor-associated factor 2

Tregs: regulatory T lymphocytes

TRIF: TIR-domain-containing adapter-inducing interferon beta

Trx: thioredoxin

Trxip: thioredoxin Trx-interacting protein

TrxSS:

UC: ulcerative colitis

VCAM: vascular cell adhesion molecule

VEGF: vascular endothelial growth factor

ZO-1: zonula occludens-1

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Table 1. Antioxidant status in human IBD patients.**Antioxidant enzymes/molecules whose activity/levels decrease with the severity of IBD**

<i>Molecule</i>	<i>Localization</i>	<i>Reference</i>
Reduced glutathione	intestinal mucosa	234
Superoxide dismutase-1	intestinal mucosa	132
Superoxide dismutase-1	red cells	136
Catalase	red cells	136
Glutathione-peroxidase-1	red cells	136
Glutathione-peroxidase-2	intestinal mucosa	256
Carotenoids	plasma	93
Vitamin C	plasma	93

Antioxidant enzymes/molecules whose activity/levels increase with the severity of IBD

Thioredoxin	intestinal mucosa	252
Aquaporin	intestinal mucosa	256

Table 2. Enhanced synthesis of inflammatory cytokines, chemokines and adhesion molecules, induced by lipid oxidation products relevant to IBD progression.

<i>Lipid oxidation product/s</i>	<i>Cell type</i>	<i>Induced molecule/s</i>	<i>Reference</i>
7K	macrophages	IL-1 β	157
7K, 7 β -OH	macrophages	IL-8	155
7K	endothelial cells	adhesion molecules	233
7K/7 α -, 7 β -OH	endothelial cells	adhesion molecules/IL-1 β	146
7 α -OH	endothelial cells	MCP-1	148
7K	smooth muscle cells	IL-6	245
7 β -OH	colon epithelial cells	IL-8	163
HNE	macrophages	TGF β 1	147
HNE	macrophages	MCP-1	189
Ox-PLs	macrophages	VEGF/IL-8/COX-2	29
Ox-PLs	macrophages	GM-CSF/IL-1 β , IL-6/IL-8/MCP-1/TNF α	275

7K: 7-ketocholesterol; 7 α -OH: 7 α -hydroxycholesterol; 7 β -OH: 7 β -hydroxycholesterol; HNE: 4-hydroxynonenal; Ox-PLs: oxidized phospholipids; IL: interleukin; TGF β 1: transforming growth factor beta1; MCP-1: monocyte chemotactic protein-1; MIP-1 β : Macrophage inflammatory protein 1 β ; VEGF: vascular endothelial growth factor; COX-2: cyclooxygenase-2; GM-CSF: Granulocyte-macrophage colony-stimulating factor; TNF α : Tumor necrosis factor-alpha.

Legends to Figures

Figure 1. Inappropriate response of the mucosa-associated immune system to commensal and pathogenic microbiota in IBD. NLRs: NOD-like receptors recognizing bacteria-derived peptidoglycan; TLRs: Toll-like receptors recognizing bacteria-derived lipopolysaccharide, flagellin or unmethylated CpG.

Figure 2. Cell types involved in Ulcerative Colitis

EC: endothelial cell; IEC: intestinal epithelial cells; iL: intraepithelial lymphocyte; M: macrophage; PMN: polymorphonuclear cells; Th: T helper lymphocyte. To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars

Figure 3. Cell types involved in Crohn's disease

DC: dendritic cell; EC: endothelial cell; GC: goblet cell; MC: Microfold cell; PC: Paneth cell; Th: T helper lymphocyte. To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars

Figure 4. Histological evidence of Crohn's disease in colonic biopsy.

Diffuse inflammation involves the greater part of the tissue section. Giant cells, histiocytes and a large number of intraepithelial lymphocytes are present. Glands appear normal but hyperplastic.

The specimen was stained with Hematoxylin and Eosin (20X magnification) and viewed using a microscope equipped with a LEICA Photocamera. To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars

Figure 5. Primary contribution of the pleiotropic cytokine TGFβ1 to the chronicization of IBD process.

Reactive oxygen species, as well as lipid oxidation products such as oxysterols and HNE, contribute to regulate the activity of TGFβ1. This cytokine is a crucial molecule produced during inflammatory

reactions. TGF β 1 is the “crossroads” for cell fate: it is responsible for enterocyte differentiation and aging, with consequent induction of cell death. In parallel, it also activates intestinal wound healing, inducing angiogenesis and fibrosis, this last mainly involved in Crohn’s disease pathogenesis.

L \cdot : lipid radical; LO \cdot : alkoxy radical; LOO \cdot : peroxy radical; H₂O₂: hydrogen peroxide; O₂ $^{\cdot-}$: superoxide; HNE: 4-hydroxynonenal.

Figure 6. Intracellular production of reactive oxygen species in intestinal epithelial cells.

ROS production was visualized in colon intestinal CaCo-2 cells using the fluorescent probe 2',7'-dichlorofluorescein derived from the cleavage and oxidation of its reduced form, dichlorofluorescein. The fluorescence, shown in green, was monitored directly by laser scanning confocal microscopy (microscope Zeiss LSM 510, Carl Zeiss SpA, Arese, Milan, Italy) equipped with a plan neofluar lens 20 \times /0.75. A: untreated cells; B: CaCo-2 cells treated with 1 mM H₂O₂. To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars

Figure 7. Role of oxysterols in regulating cell signaling pathways related to oxidative reactions.

Among various signaling pathways induced by oxysterols, the activation of Ca²⁺-dependent phospholipase A₂, followed by arachidonic acid release and NADPH oxidase up-regulation, has been hypothesized. The consequent ROS generation induces a complex network of cellular signals, which can lead to inflammation through NF- κ B or to cell death by activating mitochondrial apoptotic pathway.

Plase A₂: phospholipase A₂; AA: arachidonic acid; PYK2: proline-rich tyrosine kinase 2; PKC: protein kinase C; ERK1,2: extracellular signal-regulated kinase1,2; ROS: reactive oxygen species; NF- κ B: nuclear factor kappaB; Bim: Bcl-2 interacting mediator of cell death; Bad: Bcl-xL/Bcl-2 associated death promoter.

Figure 8. IL-1 β induction in enterocytes stimulated with oxysterols.

Stimulation of CaCo-2 enterocyte-like cells with a mixture of dietary oxysterols comprising: 42.96% 7-ketocholesterol, 32.3% 5 α ,6 α -epoxycholesterol, 5.76% 5 β ,6 β -epoxycholesterol, 4.26% 7 α -hydroxycholesterol, 14.71% 7 β -hydroxycholesterol. A: untreated cells; B: oxysterol-treated cells.

IL-1 β production was detected by red immunofluorescence, using a confocal microscope Zeiss LSM 510 (Carl Zeiss SpA, Arese, Milan, Italy) with a plan neofluar lens 40 \times /0.5. To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars

Figure 9. NF-kB target genes likely implicated in the development of IBD.

IL: interleukin; TNF: tumor necrosis factor; ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; MCP-1: monocyte chemotactic protein-1; MIP-1: macrophage inflammatory protein 1; RANTES: regulated upon activation, normal T cell expressed and secreted; PDGF: platelet-derived growth factor; EGF: epidermal growth factor; IAP: inhibitor of apoptosis, FLIP: flice-like inhibitory protein; MLCK: myosin light-chain kinase; VEGF: vascular endothelial growth factor; TGF β 1, transforming growth factor β 1; MMP: metalloproteinases; COX-2: cyclooxygenase-2; MnSOD: manganese superoxide dismutase; iNOS: inducible nitric oxide synthase.

Figure 10. Current therapeutic approaches to IBD

Figure 11. Main cellular sites of production of reactive oxygen species targeted by IBD therapy.

Membrane NADPH oxidase (NOX) and mitochondria are the two main sources of ROS generation, which target pathogen recognition pathways and activate the inflammatory response mediated by NF-kB activation. The use of molecules to control oxidative and inflammatory reactions by interfering in different ROS production sites is the most probable future approach to treating IBD, or at least delaying its progression.

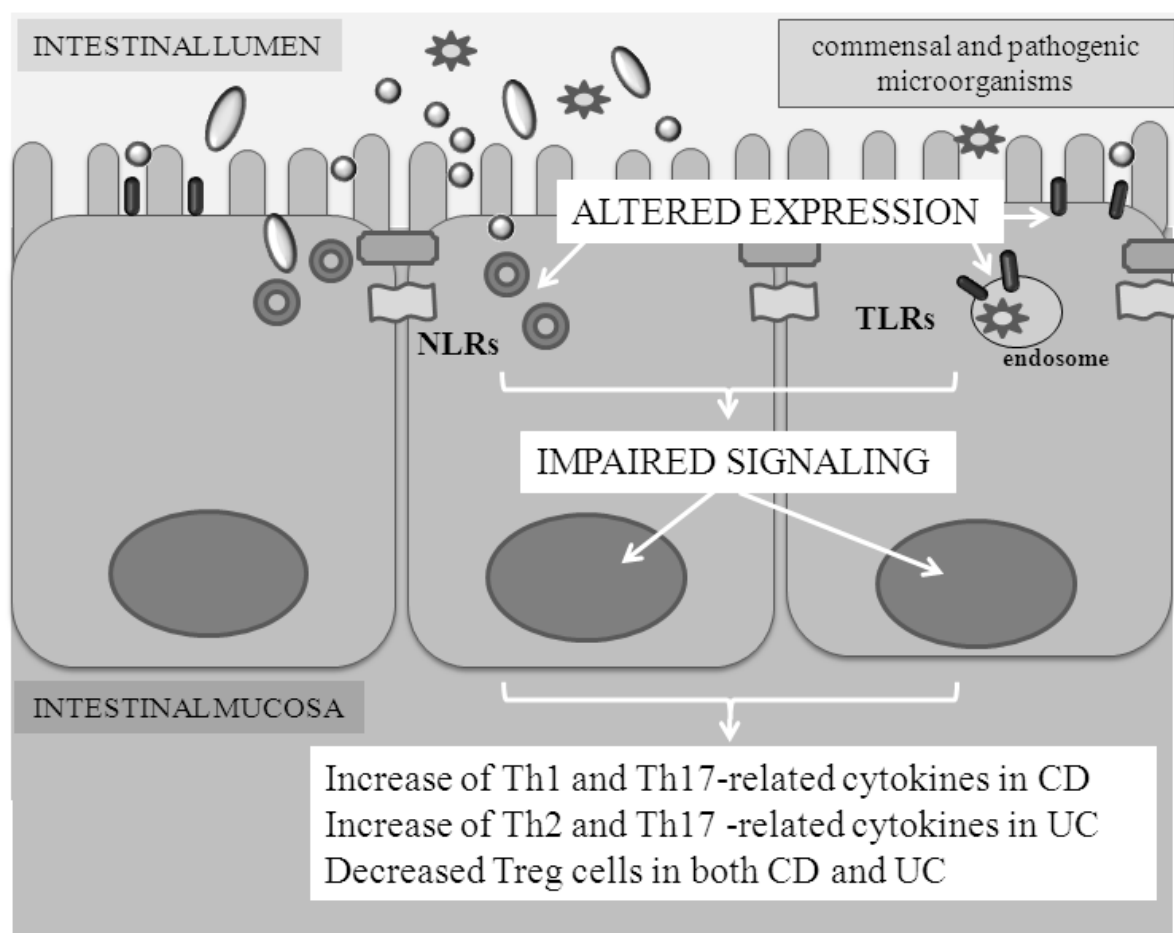


Fig.1

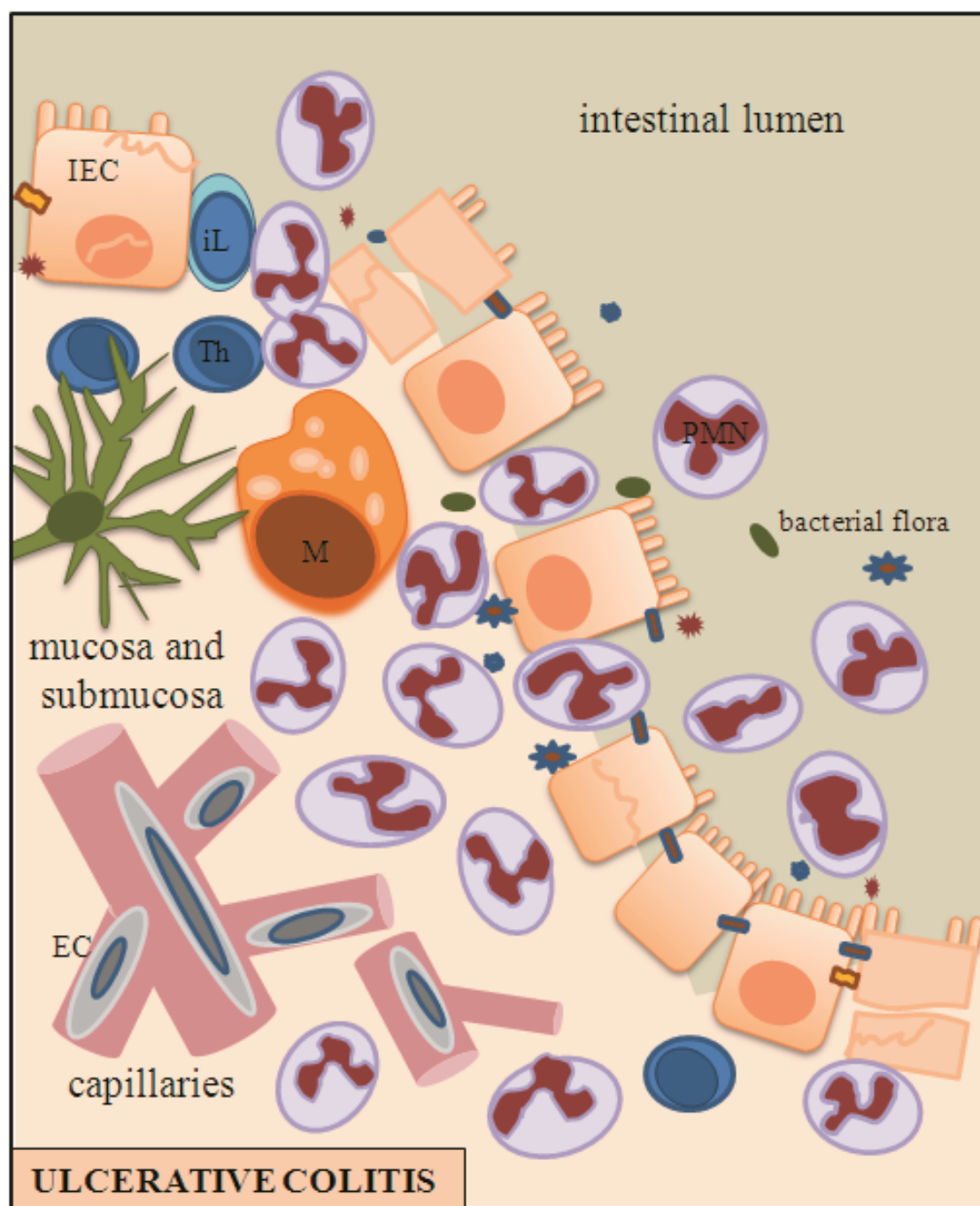


Fig.2

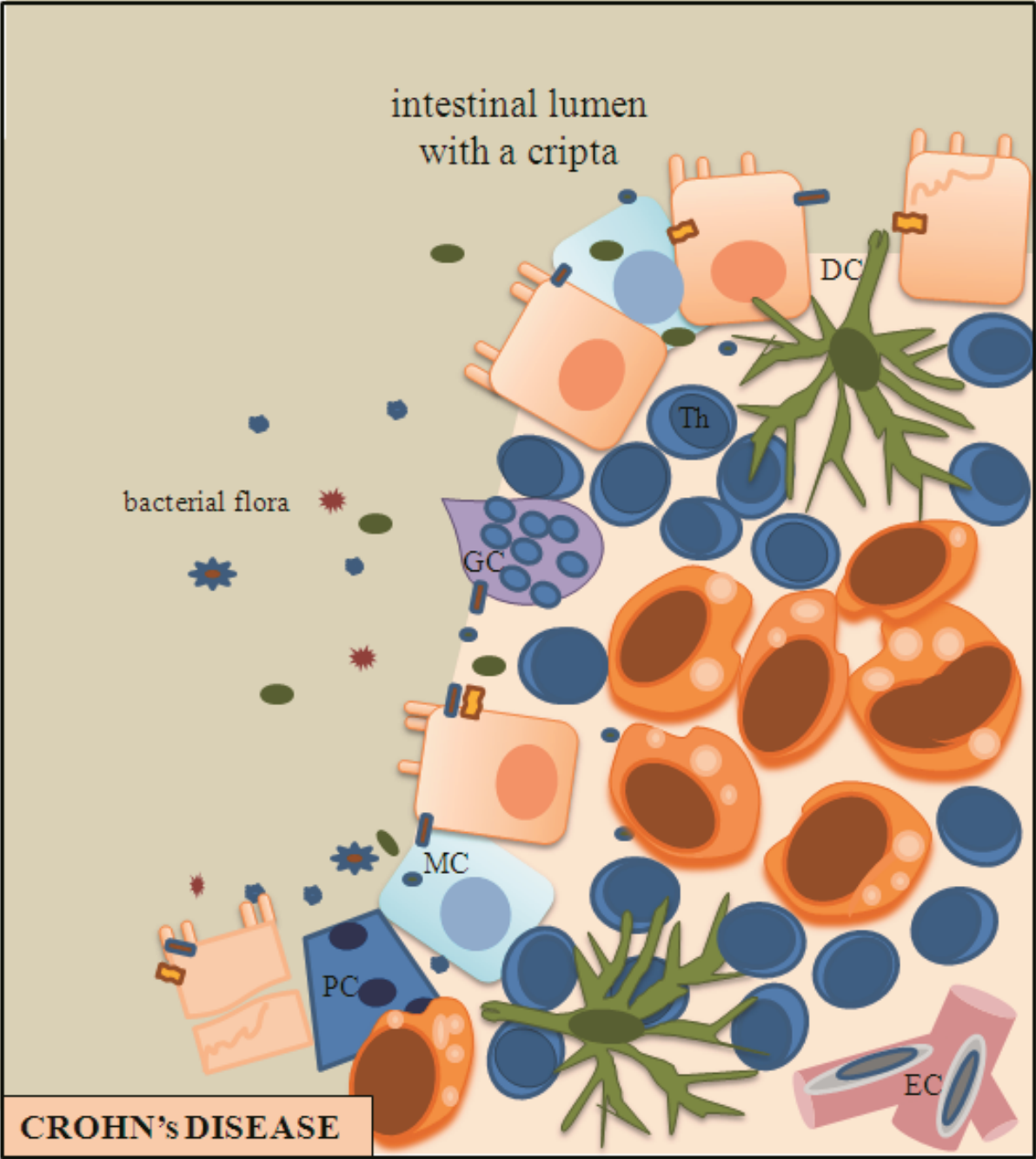


Fig 3

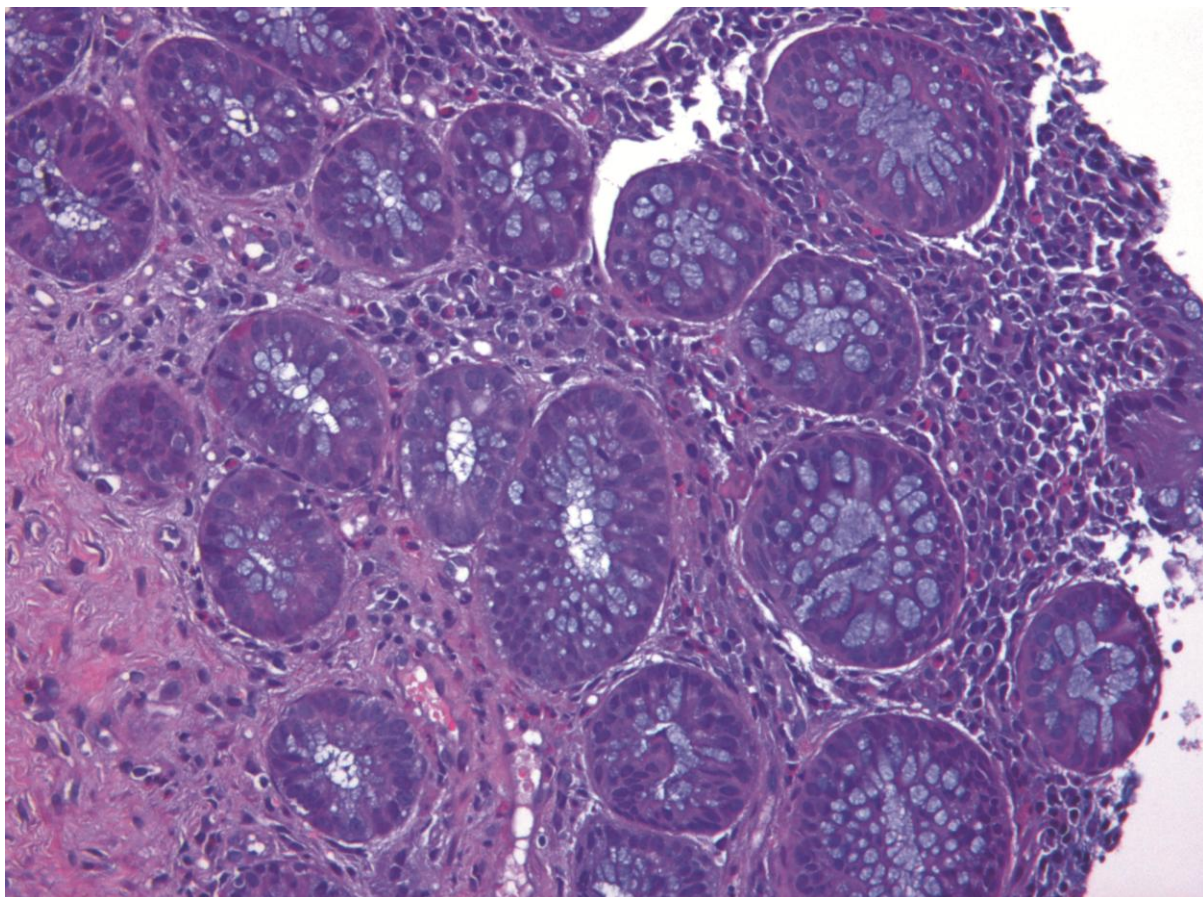


Fig 4

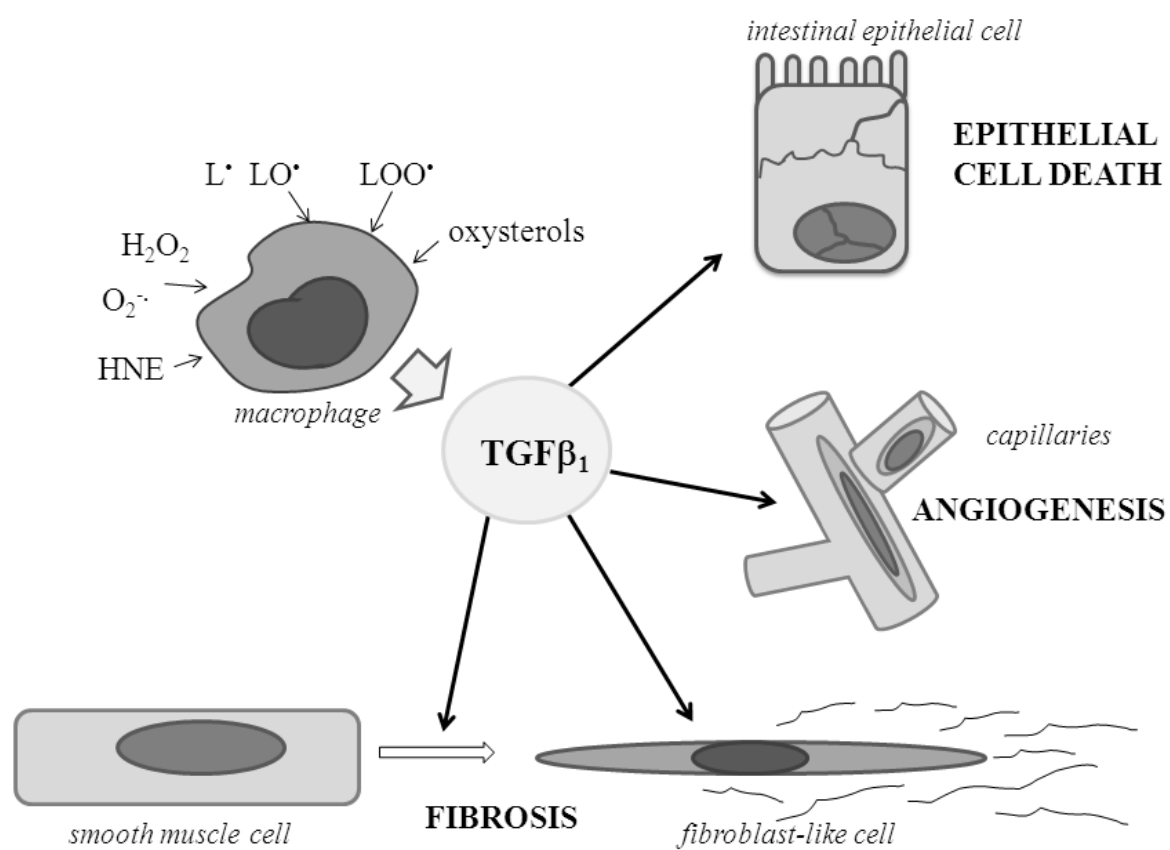


Fig 5

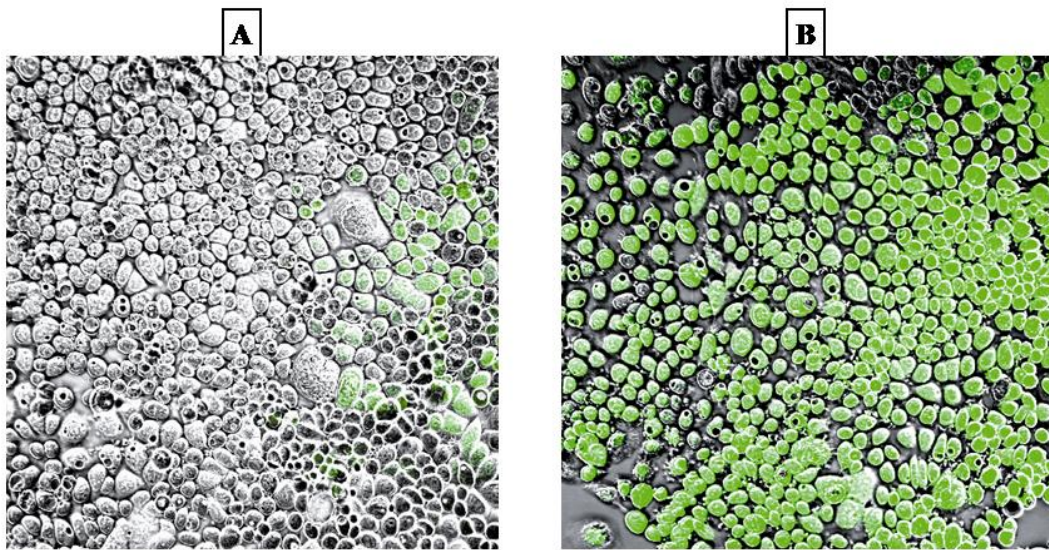


Fig 6

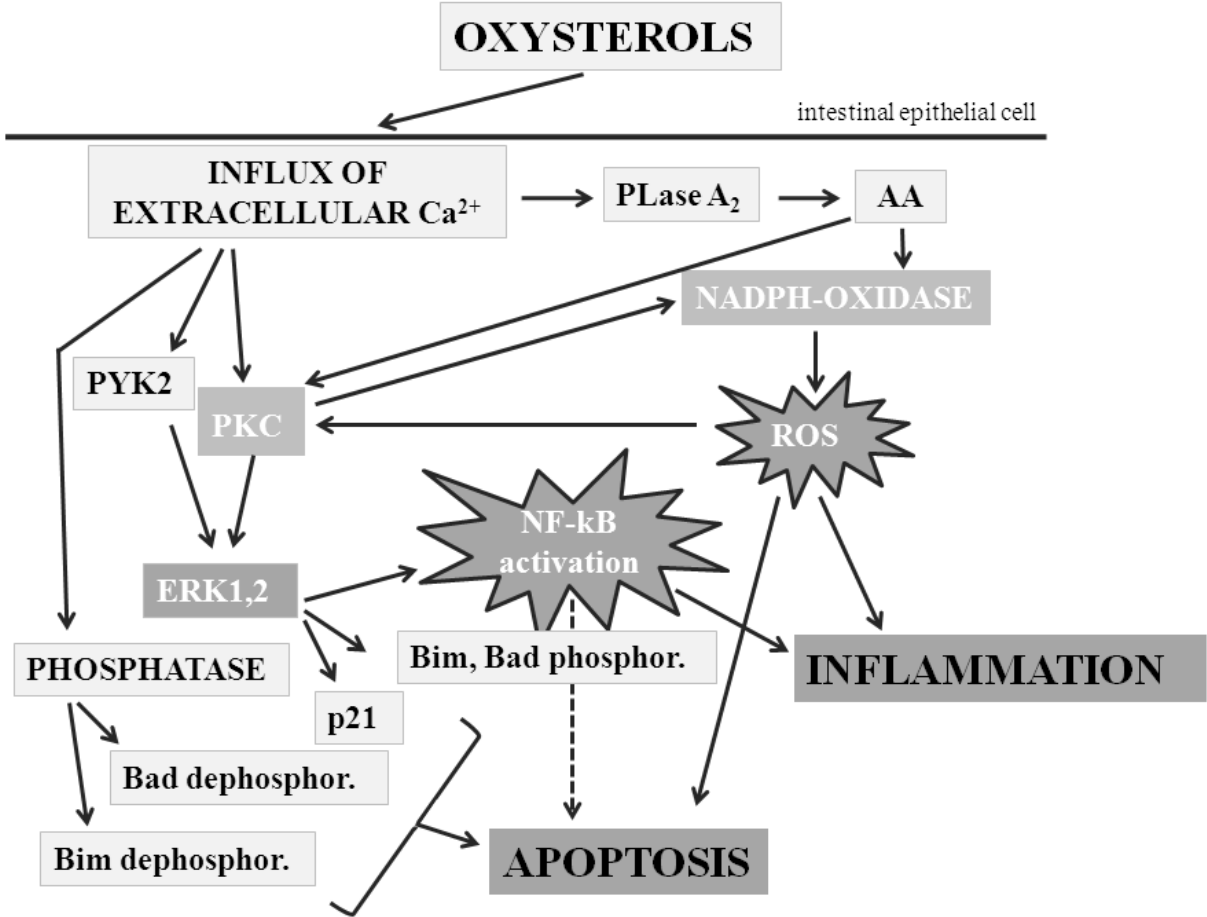
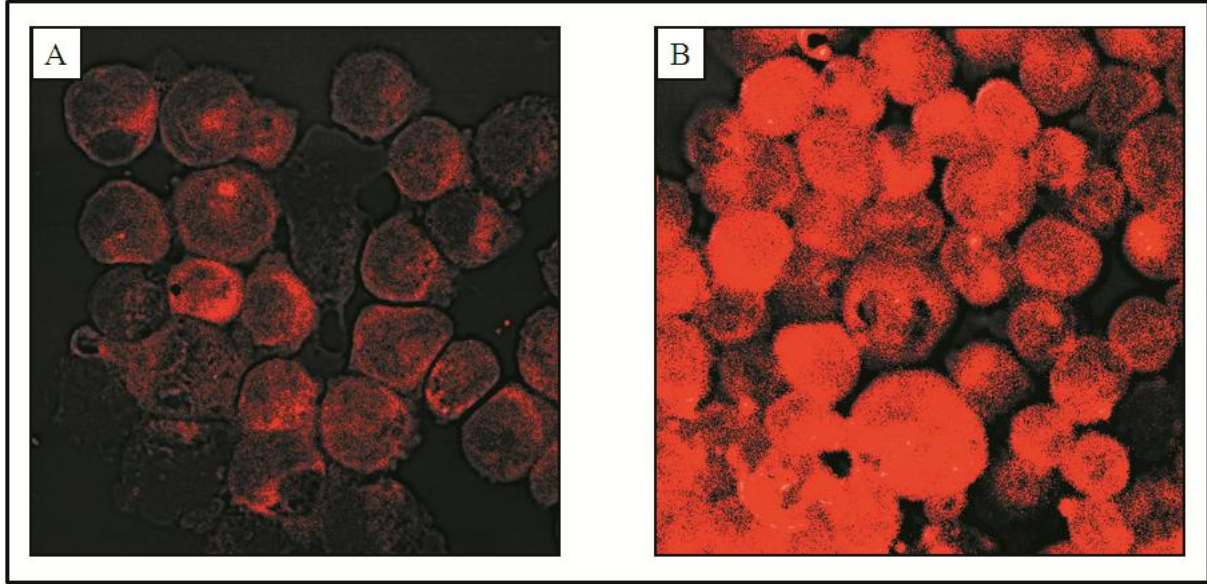
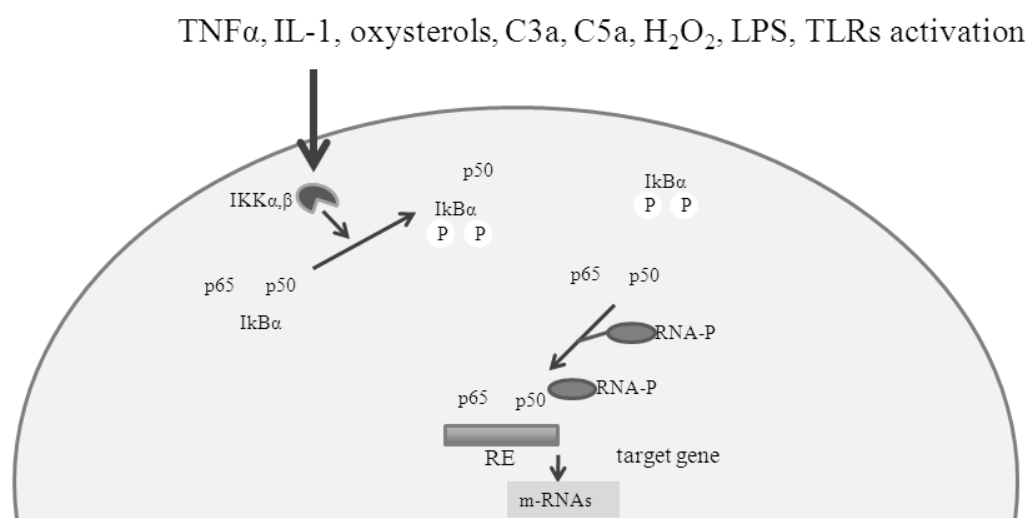


Fig 7



Fig

8

**1) pro-inflammatory cytokines, adhesion molecules, chemokines**

(IL-1 alpha, IL-1 beta, IL-2, IL-6, IL-8, IL-16, TNF α ,...), (ICAM1, VCAM1, E-selectin...), (MCP-1, MIP-1 α , MIP-1 β , RANTES...)

2) molecules regulating cell proliferation and survival

(PDGF, EGF, cyclins D1 and D3, CDK2, Bcl-xl, survivin, LAPs, FLIP...)

3) molecules regulating the permeability of the intestinal barrier and angiogenesis

(MLCK, claudins...), (VEGF, TGF β , IL-8, MCP-1, TNF α ...)

4) metalloproteinases

(MMP-1, -2, -3, -9)

5) enzymes inducing eicosanoids and reactive oxygen and nitrogen species

(phospholipase A2, COX-2, lipoxygenase, MnSOD, iNOS...)

Fig 9

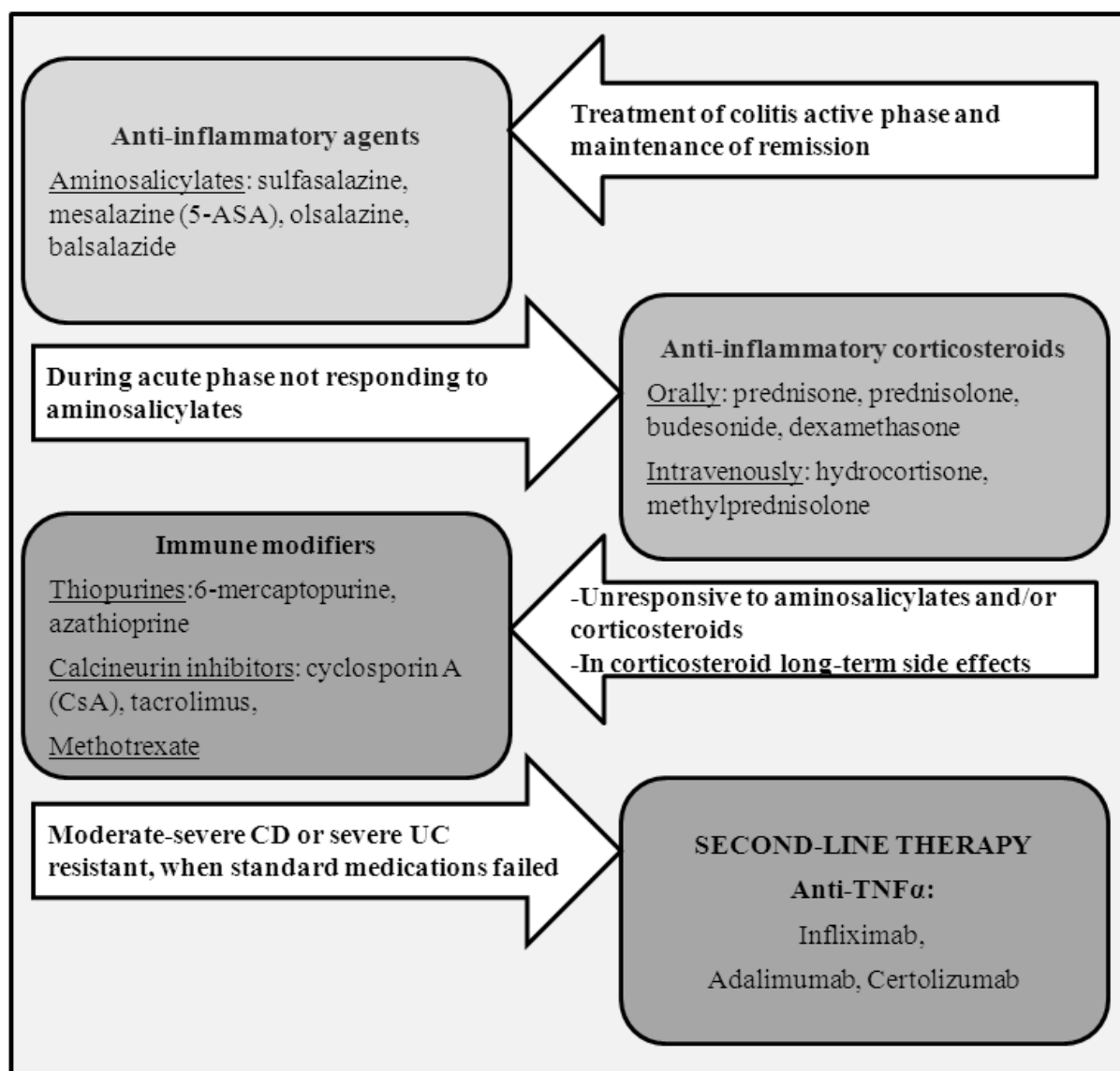


Fig 10

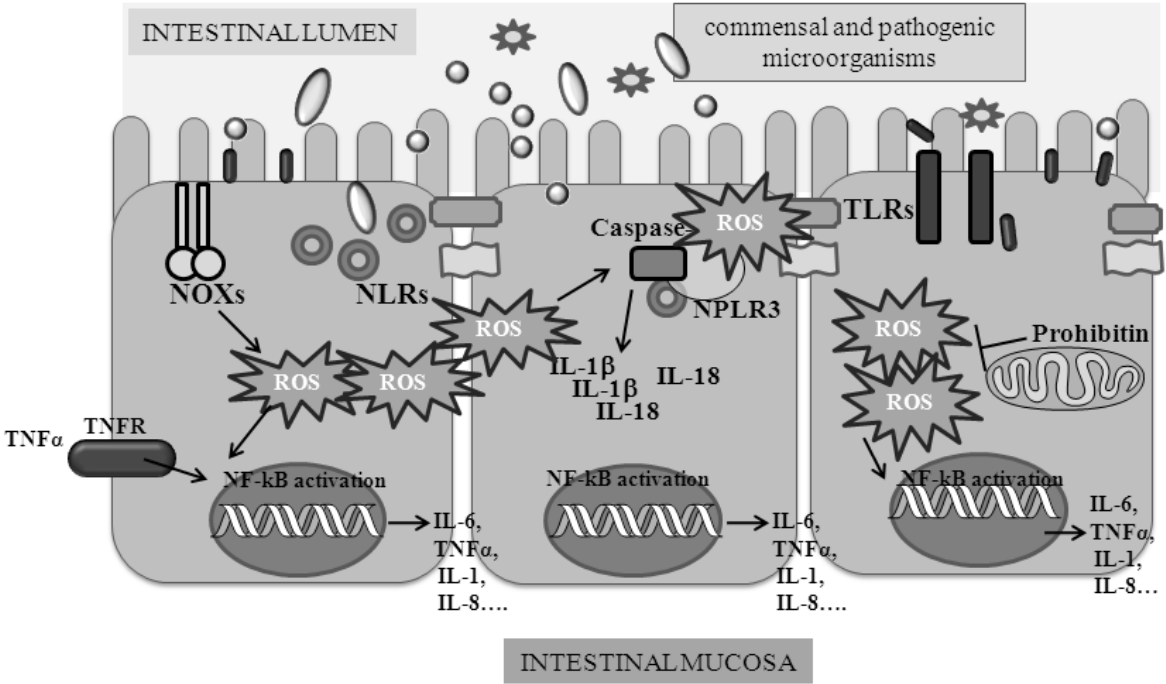


Fig 11